

Chromatin Structure of a Developmentally Regulated Single Copy Gene in *Dictyostelium discoideum*

INAUGURAL-DISSERTATION

zur Erlangung der philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von

JOVAN PAVLOVIĆ
aus Rüschegg (BE)

Begutachtet von den Herren
Prof. Dr. R.W. Parish
Prof. Dr. Th. Koller



Juris Druck + Verlag Zürich

1985

Chromatin Structure of a Developmentally Regulated Single Copy Gene in *Dictyostelium discoideum*

INAUGURAL-DISSERTATION

zur Erlangung der philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von

JOVAN PAVLOVIĆ
aus Rüschegg (BE)

Begutachtet von den Herren
Prof. Dr. R.W. Parish
Prof. Dr. Th. Koller



Juris Druck + Verlag Zürich
1985

ISBN 3 260 05108 2

CONTENTS

I. INTRODUCTION	5
II. MATERIALS AND METHODS	9
1. Cell cultures	9
2. Preparation of nuclei	9
3. Topoisomerase assay	10
4. Micrococcal nuclease digestion	10
5. Restriction enzyme digestion	11
6. Endogenous nuclease digestion	11
7. Nuclear DNA extraction	11
8. Digestion of pure DNA	11
9. Gel electrophoresis	12
10. Clones	13
11. Isolation of plasmids	13
12. Isolation of plasmid inserts	14
13. Subclone pSH209	14
14. Transfer of DNA onto DBM paper	15
15. Transfer of DNA onto nitrocellulose filters	15
16. Transfer of RNA onto DBM paper	15
17. Nick Translation	16
18. Hybridization	16
19. Isolation of total cellular RNA	17
20. Isolation of poly(A ⁺) RNA	17
21. Separation of prestalk and prespore cells	18
III. RESULTS	19
1. Restriction map of the cysteine proteinase I cDNA and genomic DNA	19
2. Chromatin structure of the coding region of the cysteine proteinase I gene	21

(a) Change in cysteine proteinase I mRNA concentration during development	21
(b) Micrococcal nuclease digestion	22
(c) Sensitivity to digestion by restriction endonucleases	23
3. Chromatin structure of three further genes transcribed by RNA polymerase II	25
(a) Clone pCZ22	25
(b) Clone pSC253	27
(c) Clone pDd812	29
4. Chromatin structure of the flanking regions of the cysteine proteinase I gene studied by indirect end-labelling	31
(a) Endogenous nuclease cleavage	32
(b) Micrococcal nuclease cleavage	34
5. Are the flanking regions of the cysteine proteinase I gene cleaved by topoisomerases?	36
 IV. DISCUSSION	 65
1. Micrococcal nuclease digestion of coding regions	65
2. Accessibility of the coding region of the cysteine proteinase I to restriction endonucleases	66
3. Endogenous nuclease and micrococcal nuclease hypersensitive sites flanking the cysteine proteinase I gene	67
4. A model of cysteine proteinase I gene regulation	68
 V. SUMMARY	 74
ZUSAMMENFASSUNG	76
 VI. REFERENCES	 78
 VII. ABBREVIATIONS	 83

I. INTRODUCTION

An understanding of the control of gene expression in eukaryotes will depend in part on knowledge of the chromatin structure of active genes and their coding regions. However, there is still controversy regarding the presence or absence of nucleosomes in transcribing chromatin and, although correlations between certain features of chromatin structure and gene expression have been found, their causal relationship is unclear (Mathis et al., 1980; Cartwright et al., 1982; Igo-Kemenes et al., 1982; Weisbrod, 1982; Butler, 1983).

Evidence is accumulating that the nucleosomes associated with the coding region of transcribing ribosomal genes take on an altered structure or may dissociate from the DNA (Labhart et al., 1983; Ness et al., 1983; Palen and Cech, 1983; Davis et al., 1983; Prior et al., 1983). The active genes are particularly sensitive to micrococcal nuclease. We found a continuous distribution of rDNA fragments was produced following digestion of Dictyostelium discoideum nucleoli with micrococcal nuclease (Labhart et al., 1983; Ness et al., 1983) or methidiumpropyl-EDTA-iron(II) (Parish et al., 1985). The loss of periodicity may result from changes in nucleosome structure but also from an undefined linker length between the nucleosomes. However, electron microscopy data showed strongly transcribed rDNA contains rows of transcription complexes so tightly packed that nucleosomes could not form between them (Ness et al., 1983; Labhart et al., 1984; Sogo et al., 1984). Although nucleosomes preclude extensive crosslinking of DNA with

trimethylpsoralen, crosslinking in the coding region was extensive in highly active ribosomal genes without disruption of the transcription complexes (Sogo et al., 1984). Moreover, treatment with psoralen in the presence of 2 M NaCl increased crosslinking in the nucleosome-containing inactive chromatin but not in the coding region (Sogo et al., 1984). Finally, the coding region of actively transcribing genes was accessible to restriction enzymes in contrast to the inactive spacer (Ness et al., 1983).

Micrococcal nuclease studies on Drosophila 5S RNA genes suggested two discrete nucleosome formats exist, in one of which the center of the gene was exposed (Louis et al., 1980). The center DNA segment is required for promoting transcription (Bogenhagen et al., 1980). Similar results were obtained with Xenopus 5S RNA genes (Gottesfeld and Bloomer, 1980). Eukaryotic tRNA genes also have an internal promoter (Hofstetter et al., 1981). Nucleosome phasing disappeared when Xenopus tRNA genes were transcriptionally active, but no preferential exposure of the promoters was observed (Bryan et al., 1982). However, DeLotto and Schedl (1984) found the internal promoter sequences of Drosophila tRNA genes were preferentially exposed to micrococcal nuclease and DNase I in chromatin.

Biochemical studies on the chromatin structure of genes transcribed by RNA polymerase II have produced conflicting results (reviewed in Reeves, 1984; Levy and Noll, 1981; Lohr, 1981; Lohr, 1983; Anderson et al., 1983). Discrete DNA segments in the 5' flanking region of several genes show a very high sensitivity to endonuclease attack. These hypersensitive sites have been observed adjacent to genes encoding heat-shock proteins (Wu, 1980), β -globin (Stalder et al., 1980; McGhee et al., 1981), histones (Samal et al., 1981), a Drosophila glue protein (Shermoen and Beckendorf, 1982) and

on SV40 minichromosomes (Saragosti et al., 1980). Furthermore, these sites have been associated with many different sequences which are involved in gene expression and frequently their appearance has been correlated with developmental and tissue-specific gene activation (Stalder et al., 1980; Keene and Elgin, 1981; McGhee et al., 1981; Cartwright et al., 1982; Sweet et al., 1982; Shermoen and Beckendorf, 1982; Cereghini et al., 1983; Kaye et al., 1984; Fritton et al., 1984). DNA sequences related to nuclease hypersensitive sites include promotor regions within approximately 200 bp upstream of the transcription start (Wu et al., 1980; Keene and Elgin, 1981; McGhee et al., 1981; Cartwright et al., 1982). The whole promotor region of the thymidine kinase gene, for example, becomes hypersensitive to DNase I and several restriction enzymes when the gene is expressed (Sweet et al., 1982; McKnight and Kingsbury, 1982). Sequences further upstream from the transcription start are also found to be nuclease hypersensitive. The developmentally regulated expression of the Sgs4 gene of Drosophila is controlled by a sequence located between -330 bp and -550 bp upstream of the transcription start, which becomes accessible to DNase I when the gene is activated (Shermoen and Beckendorf, 1982). These nuclease hypersensitive sites may represent nucleosome-free gaps up to 200 bp in length which facilitate interaction with the transcription machinery (McGhee et al., 1981; Worcel et al., 1983; Udvardy and Schedl, 1984). Hypersensitive sites associated with transcription are often found considerable distances upstream of the cap site (Chung et al., 1983; Burch and Weintraub, 1983; Kaye et al., 1984; Fritton et al., 1983). Enhancer elements exhibit also DNase I hypersensitivity at or near them. The 72 bp repeat enhancer of the SV40 minichromosome is hypersensitive to DNase I (Saragosti et al., 1982; Cereghini et al., 1983). Even translocation of the enhancer 3.5 kb upstream preserved its function and DNase I hypersensitivity (Jongstra et al., 1984). The enhancer elements of the genes coding

for the immunoglobulin κ light chain and μ heavy chain are located downstream of the transcription start (Gilles et al., 1983; Banerji et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984) and are related to a region of DNase I hypersensitivity (Mills et al., 1983; Parslow and Granner, 1982; Weichet et al., 1982; Chung et al., 1983; Picard and Schaffner, 1984).

In the studies reported here we have analyzed the chromatin structure of a single copy gene in D.discoideum. The gene is developmentally regulated, messenger RNA accumulation commencing at the 6th hour of development and reaching a maximum between 8 and 12 h when it constitutes about 1% of the mRNA population (Bogdanovsky-Sequeval et al., 1984; Williams et al., 1985). The messenger RNA can be accumulated prematurely when exogenous cAMP is present (Williams et al., 1980). It is approximately 1200 nucleotides in length and encodes a 36,000 dalton polypeptide (cysteine proteinase I) with a high degree of homology to plant and animal cysteine proteinases (Williams et al., 1985). We have used micrococcal nuclease digestion to detect changes in chromatin structure of the coding region when the gene is transcribed. Using the indirect-endlabelling technique (Wu, 1980; Nedospasov and Georgiev, 1980) we have mapped the micrococcal nuclease hypersensitive sites at the 5' and 3' flanking regions of the gene. The results suggest a three state model for changes in chromatin structure associated with cysteine proteinase I activation and transcription.

II. MATERIALS AND METHODS

1. Cell cultures

D. discoideum cells, axenic strain Ax2, were grown in shaking liquid culture with HL-5 medium (Cocucci and Sussman, 1970). Differentiation was induced by washing the cells with K/PO₄ buffer (20 mM potassium phosphate buffer, pH 6.2) and resuspending about 4.5×10^9 cells in 25 ml of the same buffer. The 25 ml cell suspension was spread on 1.5% agarose (Fluka) in K/PO₄ in 28 x 48 cm aluminium trays. Each tray contained 400 ml agarose and the final cell concentration was 3.5×10^6 cells/cm². When almost dry, two trays were placed face to face and incubated in the dark at 21°C for 2, 4, 6, 8, 12, 15 or 18 h. Cells were harvested from the early differentiation stages (2-8 h) by washing the cells from the agarose with cold K/PO₄ buffer, disaggregated by squeezing through a 20 ml syringe (0.8 x 80 mm needle) and washing with buffer. Cells were harvested from the later differentiation stages (12-18 h) by washing the aggregates with cold K/PO₄ buffer onto nylon filters (63 µm mesh, "Nybolt -25", Swiss Bolting Cloth MFG Co. Ltd). Non-aggregated cells wash through the filter. The aggregates were scraped into buffer and disaggregated by squeezing them through a syringe.

2. Preparation of nuclei

Nuclei used in all experiments (except for the topoisomerase assay) were isolated as previously described (Charlesworth and Parish, 1977; Widmer et al., 1979; Ness et al., 1983) with the following modifications: lysis with Triton X-100 was

carried out at 0°C; the nuclear pellet was washed in 20 mM Tris-HCl, pH 7.5, 0.5 mM CaCl₂.

Nuclei to be used in the topoisomerase experiments were obtained by washing cells with NB buffer (10 mM Tris-HCl, pH 7.2, 3 mM CaCl₂, 1 mM MgCl₂, 100 mM sucrose). The cells (3×10^9) were taken up in 200 ml NB buffer and lysed by adding 0.3% Nonidet P-40 and stirring on ice. Nuclei were obtained by centrifuging at 2000 g for 5 min at 4°C (Gocke et al., 1983).

3. Topoisomerase assay

Nuclei were taken up in 50 ml NB buffer and divided into two 25 ml aliquots. Both aliquots were incubated for 2 min at 25°C. One aliquot was placed in ice and NaCl and EDTA, final concentrations 0.8 M and 10 mM respectively, were added. Incubation was for 5 min on ice and sodium dodecyl sulphate then added to a final concentration of 1%. To the second aliquot 1% sodium dodecyl sulphate and 10 mM EDTA were added, the solution incubated for a further minute; then placed on ice and NaCl (final concentration 0.8 M) added (Gocke et al., 1983). Both samples were incubated with 250 µg/ml proteinase K for 2 h at 37°C.

4. Micrococcal nuclease digestion

Washed nuclei (1.5×10^9 per ml) were resuspended in 3 ml 20 mM Tris-HCl, pH 7.8, 0.5 mM CaCl₂ and incubated at 25°C with either 80 units/ml (nuclei from vegetative cells) or 50 units/ml (differentiating cells) micrococcal nuclease (Widmer et al., 1979). Incubation times varied from 15 s to 5 min. The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulphate to 0.5%. The samples were treated with 250 µg/ml proteinase K for 2 h at 37°C.

For the indirect end-labelling experiments, nuclei from vegetative or differentiating cells were treated with 12.5 units/ml micrococcal nuclease.

5. Restriction enzyme digestion

Nuclei (10^8 per ml) were resuspended in 0.5 ml 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT and incubated for 30 min at $37^\circ C$ with 200 units of either EcoRI, HindIII or HinfI. The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulphate to 0.5%. The lysate was treated with proteinase K.

6. Endogenous nuclease digestion

Nuclei (1.5×10^9 per ml) were resuspended in 10 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and incubated for 20 min at $25^\circ C$ or $0^\circ C$. The reaction was stopped with 20 mM EDTA and 0.5% sodium dodecyl sulphate and followed by a treatment with proteinase K.

7. Nuclear DNA extraction.

Proteinase K treated nuclear lysates were extracted twice with one volume of phenol/chloroform/isoamylalcohol (25/24/1, by volume), then extracted once with one volume of chloroform/isoamylalcohol (24/1). DNA was precipitated with 2 volumes of ethanol (1 h, $-70^\circ C$), the precipitates washed once with 80% ethanol, resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 $\mu g/ml$ RNase A and incubated for 60 min at $37^\circ C$. DNA was ethanol precipitated and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE-buffer).

8. Digestion of pure DNA

Pure DNA was cut with micrococcal nuclease at $0^\circ C$; 20 μg of DNA in 200 μl of 20 mM Tris-HCl, pH 7.8, 0.5 mM $CaCl_2$ was

incubated with 10 units of enzyme for 60 s. Pure DNA was cut with restriction enzymes according to instructions of the suppliers.

9. Gel electrophoresis

DNA fragments were analyzed on horizontal gels 25 cm x 20 cm x 0.3 cm. Gels contained between 1% and 2.3% Sigma type II agarose. The electrophoresis buffer was 36 mM Tris, 30 mM NaH_2PO_4 , 1 mM EDTA (pH 7.8). Gels were run for 16 h at 4°C, 40 V. When DNA fragments were to be isolated, Pharmacia agarose NA was used and the electrophoresis buffer was 50mM Tris, 20 mM sodium acetate, 2mM EDTA (pH 7.8). Gels were stained for 30 min in 1 µg/ml ethidium bromide and photographed under UV light (254 nm).

RNA was analyzed on horizontal gels containing 1.5% agarose and 6% (v/v) formaldehyde. The electrophoresis buffer was 20 mM sodium phosphate buffer (pH 7.6), 3% formaldehyde. RNA was solubilized in 50% deionized formamide, 6% formaldehyde, 20 mM sodium phosphate buffer (pH 7.6) and incubated for 15 min at 55°C. After rapid cooling, a 1/10 volume of 50% glycerine, 0.025% bromophenol blue was added. Electrophoresis was at 30 V for 16 h at room temperature; the electrophoresis buffer was continuously recycled (Rave et al., 1979; Schwinghamer and Shepherd, 1980).

Single stranded DNA fragments were separated on horizontal alkaline agarose gels containing 1.5% agarose, 30 mM NaOH, 1 mM EDTA using 30 mM NaOH, 1 mM EDTA as electrophoresis buffer. DNA samples to be analysed were dissolved in alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% Ficoll type 400, Pharmacia, and 0.025% bromocresol green). Electrophoresis was carried out at 30 V for 16 h at room temperature, recycling continuously the electrophoresis buffer (Maniatis et al., 1982).

10. Clones

pDdl1.7.10: cDNA clone of the cysteine proteinase I gene in D.discoideum (Williams and Lloyd, 1979; Williams et al., 1985).

pDd812: cDNA clone of the discoidin I gene (Williams et al., 1979; Tsang et al., 1981; Devine et al., 1982). pDdl1.7.10 and pDd812 were a gift from J.G. Williams.

pCZ22: cDNA clone of a constitutively expressed, multiple copy gene in D.discoideum (Zuker and Lodish, 1981).

pSC253: genomic clone of a developmentally regulated single copy gene in D.discoideum (Zuker and Lodish, 1981; Chung et al., 1983).

pCZ22 and pSC253 were a gift from H.F. Lodish.

11. Isolation of plasmid DNA

Transformation of E.coli strain HB101 with plasmid DNA was performed by the calcium chloride procedure. Growth of bacteria and amplification of plasmids was carried out according Maniatis et al. (1982). 500 ml bacterial culture were sedimented and washed once in TE-buffer, pH 8.0. They were resuspended in 7 ml of 50 mM Tris-HCl, pH 8.0, and 1.2 ml (10 mg/ml) lysozyme (Boehringer Mannheim) by swirling gently on ice for 5 min. After adding 1.2 ml of 0.5 M EDTA, pH 8.0, the bacterial solution was incubated on ice for another 5 min. The bacteria were lysed by adding 10.6 ml bacterial lysis buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA and 0.1% Triton X-100) standing on ice for 10 min and centrifuged at 20,000 g for 60 min at 4°C. To denature the DNA the supernatant was brought to pH 12.5 with 3 M NaOH and stirred at 150 rpm for 10 min. The circular plasmid DNA was renatured by lowering the pH with 1 M Tris-HCl (pH 7.5) to pH 8.5 while bacterial genomic DNA stayed denatured. Following stirring for 3 min NaCl was added to a final concentration of 0.5 M. The supernatant was extracted once with one volume of phenol (equilibrated with 3% NaCl) and once with one volume of chloroform. The precipitated and washed pellet was

resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 µg/ml RNase A and incubated for 30 min at 37°C. Prior to precipitating the plasmid DNA with 0.55 volumes of isopropanol for 15 min at room temperature, sodium acetate to a final concentration of 0.25 M was added. The washed and resuspended DNA was reprecipitated from 0.25 M sodium acetate with two volumes of ethanol. The plasmid DNA was stored in TE-buffer, pH 8.0, at -20°C.

12. Isolation of plasmid inserts

50 - 100 µg plasmid DNA were digested with the appropriate restriction enzymes followed by fragment separation on 1.5-2.0% agarose gels and staining with 0.5% ethidium bromide. A strip of DEAE cellulose membrane (Schleicher and Schuell Na 45) was placed in an incision just ahead of the band to be recovered. Electrophoresis was continued until binding was complete. To remove residual agarose the membrane was washed in low salt buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 150 mM NaCl). The bound DNA was eluted by incubating the strip in high salt buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1.0 M NaCl) for 30 min at 65°C. After washing the membrane with a small volume of the same buffer the eluted DNA was precipitated with 2.5 volumes of ethanol. Reprecipitation was from 0.25 M sodium acetate to remove residual NaCl.

13. Subclone pSH209

Vector pUC12 was cut with SmaI and HindIII excising a 30 bp DNA fragment in the polylinker. The two fragments were separated on a 1% agarose gel and the linearized plasmid was recovered by using a DEAE-cellulose membrane as described above. The 209 bp ScaI - HindIII cDNA fragment of plasmid pDdl1.7.10 and the linearized pUC12 were ligated over night at 4°C followed by transformation of E.coli strain HB101 (Maniatis et al., 1982). Screening of the bacterial colonies was performed according Maniatis et al. (1982).

Ampicillin resistant colonies were grown on nitrocellulose filters laid on surfaces of agar plates and lysed with alkali. The clones were hybridized with the 209 bp ScaI - HindIII cDNA fragment.

14. Transfer of DNA onto DBM paper

DBM paper was prepared according to Alwine et al. (1979) using N-(3-nitrobenzyl-oxymethyl)-pyridinium chloride (BDH). The DBM paper was activated immediately prior to the transfer. Gels were soaked for 10 min at room temperature in 0.25 M HCl followed by 0.5 M NaOH for 60 min to denature the DNA. Gels were neutralized by incubation in 1 M sodium acetate (pH 5.0), once at room temperature and a second time at 4°C. The DNA was transferred to DBM paper by the method Southern (1975) using 1 M sodium acetate (pH 5.0) as transfer buffer and blotted overnight.

15. Transfer of DNA onto nitrocellulose filters

Nitrocellulose filters were used for the indirect end-labelling experiments. DNA was hydrolyzed by incubating the gels for 10 min in 0.25 M HCl. Denaturing of the DNA was performed in 0.5 M NaOH, 1.5 M NaCl for 60 min followed by neutralizing the gel for 1 h in 1 M Tris-HCl pH 7.5, 1.5 M NaCl. The DNA was blotted onto a nitrocellulose filter using 20 x SSC as transfer buffer. The steps were carried out at room temperature. After rinsing in 10 x SSC the filters were dried and baked for 2 h at 80°C in a vacuum oven.

16. Transfer of RNA onto DBM paper

Agarose gels containing RNA were incubated in water for 5 min at 60°C and then rapidly cooled to room temperature in water. The RNA was hydrolyzed by incubating the gels for 20 min in 50 mM NaOH at room temperature. Gels were soaked for 15 min at room temperature in 1 M sodium acetate buffer (pH 5.0) and once in cold (4°C) blotting buffer (1 M sodium acetate, pH 5.0) for 15 min.

Transfer of RNA to DBM paper was as described for DNA (Rave et al., 1979).

17. Nick Translation

Plasmids and DNA fragments (200 bp and more) were labelled with [α - 32 P] dCTP (3000 Ci/mmmole, Amersham) using an Amersham nick translation kit. Specific activities of up to 10^9 cpm/ μ g DNA were obtained.

18. Hybridization

DBM paper was prehybridized by incubating overnight at 68°C in 6 x SSC (1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate), 1 x Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 1% glycine, 0.1% sodium dodecyl sulphate, 15 mM sodium phosphate buffer (pH 7.0) and 250 μ g/ml denatured calf thymus DNA. The DBM paper was hybridized with about 50 ng of nick-translated DNA (approx. 4×10^7 cpm) in 5 ml 6 x SSC, 1 x Denhardt's, 1 mM EDTA, 0.2% sodium dodecyl sulphate, 15 mM sodium phosphate buffer (pH 7.0) and 250 μ g/ml denatured calf thymus DNA for 20 h at 68°C. Following hybridization of DNA or RNA filters, they were washed six times with 0.5 x SSC, 0.1% sodium dodecyl sulphate, 1 mM EDTA for 30 min at 68°C. The filters were exposed with 1 or 2 tungsten intensifier screens (Ilford) and X-ray film (Fuji). To reuse the DBM paper it was stripped three times for 1 min in boiling water and prehybridized as described above.

The nitrocellulose filters were prehybridized in 20 ml 6 x SSC, 5 x Denhardt's, 15 mM Na₂PO₄-buffer pH 7.0, 1% N-lauroylsarcosine, 0.1% sodium dodecyl sulphate, 10% dextran sulphate and 250 μ g/ml denatured calf thymus DNA at 68°C overnight. Hybridization was carried out for 16 h at 68°C in 10 ml of the same solution containing 1 μ g/ml 32 P labelled excised plasmid insert

(10^9 cpm/ μ g DNA). Following hybridization, the nitrocellulose filters were washed two times in 0.5 x SSC, 0.1% sodium dodecyl sulfate for 5 min at room temperature and three times for 30 min at 68°C. The filters were exposed as described for the DBM paper.

19. Isolation of total cellular RNA

Cells were isolated from growing and differentiating cultures as previously described and washed in K/PO_4 buffer. The cells were lysed in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 1% sodium dodecyl sulphate and 250 μ g/ml proteinase K (about 5×10^7 cells/ml) and incubated for 60 min at 37°C. Nucleic acids were extracted as described above; precipitated with 2.5 volumes of ethanol, washed with 80% ethanol and taken up in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 300 mM NaCl and 100 μ g/ml DNase I (Boehringer, Grade 1). Following incubation for 60 min at room temperature, sodium dodecyl sulphate to 0.5% and 250 μ g/ml proteinase K were added. After a further incubation for 60 min at room temperature, phenol extraction was performed as above. RNA was dissolved in water (gel electrophoresis) or binding buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% sodium dodecyl sulphate) for poly(A⁺) RNA isolation.

20. Isolation of poly(A⁺) RNA

Total cellular RNA in binding buffer was passed through a 0.5 ml column of oligo (dT) cellulose (BRL). After extensive washing with binding buffer the bound fraction of mRNA was eluted with four column volumes of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% sodium dodecyl sulphate). The pooled poly(A⁺) RNA was precipitated and resuspended in water.

21. Separation of prestalk and prespore cells

Slugs (15 h cell aggregates) were disaggregated in K/PO_4 buffer, 20 mM EDTA with a pasteur pipette (10^8 cells/ml). Following centrifugation, the cells were resuspended in the same buffer (4×10^8 cells/ml). Density gradients were prepared by mixing Percoll (Pharmacia) with 400 mM potassium phosphate buffer, 13% NaCl, 40 mM EDTA (Percoll: buffer solution; 95:5 v/v). About 10^8 cells were mixed with 12 ml of the density gradient solution and centrifuged in 15 ml Corex tubes for 30 min at 18,000 g and 4°C in a fixed angle rotor. The lighter prestalk cells and the denser prespore cells were collected, the cell suspensions diluted with at least 4 volumes of K/PO_4 buffer, 0.8% NaCl, and washed once with the same buffer.

III RESULTS

1. RESTRICTION MAP OF THE CYSTEINE PROTEINASE I cDNA AND GENOMIC DNA

To characterize the cysteine proteinase I gene we wished to determine the copy number per genome by Southern blot analysis. Further, we wanted to identify and localize introns by comparison of the restriction fragment sizes in cDNA and genomic DNA. This task was simplified as the cDNA of the cysteine proteinase I gene has recently been sequenced by Williams et al. (1985) and a complete restriction map was available. Fig. 1 shows the cDNA clone pDdl1.7.10 of the cysteine proteinase I gene and the excised cDNA fragments used in Southern blot analysis (Fig. 2). The plasmid Ddl1.7.10, containing a 1000 bp insert of cDNA complementary to mRNA of the cysteine proteinase I gene, was cut with the appropriate restriction endonucleases, the DNA fragments were size fractionated on 2 % agarose gels, and recovered using DEAE-cellulose membranes. This method resulted in very pure DNA fragments and no contamination with other insert fragments was observed (see Fig. 2a-c eg. lanes A1).

The 209 bp HindIII - ScaI cDNA fragment of pDdl1.7.10 was subcloned, using pUC12 as a vector (Fig. 3). pUC12 was cut with SmaI and HindIII, excising a 30 bp DNA fragment in the polylinker. The two fragments were separated on a 1% agarose gel and the linearized plasmid was recovered using a DEAE-cellulose membrane. The 209 bp HindIII - ScaI cDNA fragment and the linearized plasmid were ligated at 4°C.

Nuclear DNA of vegetative cells was cut with the restriction endonucleases indicated in Fig. 2, size fractionated on 1.5% or 2.3% agarose gels and transferred to DBM paper. The blots were hybridized with the isolated cDNA fragments of pDdl1.7.10 (Fig. 2). The Southern blot analysis of the cysteine proteinase I (Fig. 2) showed that the restriction enzymes HaeIII (He) and KpnI (K) (Fig. 2e, lanes He and K) have no cleavage sites within the gene. AvaII (A), BglII (B), Hind III (H) and HinfI (Hf) (Fig 2a-e, lanes A, B, H and Hf) cut once, AluI twice (Fig. 2a-c, lanes Al) and EcoRI three times within the coding region. When the restriction map of the genomic DNA was compared with that of the cDNA clone (Fig. 5), no additional restriction sites were detected by Southern blot analysis under the hybridization conditions used. This indicated that the cysteine proteinase I is a single copy gene. The additional bands occurring after cleavage with HinfI (Fig 2a-c, lanes Hf) are due to incomplete digestion of the genomic DNA.

The determination of the fragment sizes of the genomic DNA (Fig. 2) resulted in the restriction map shown in Figs. 4 and 5. Comparison of the fragment sizes in cDNA and genomic DNA (Fig. 5) indicated the presence of at least two introns in the transcribed region. The genomic 5' EcoRI - EcoRI fragment is 475 bp in length, 195 bp longer than its complementary cDNA sequence. The 280 bp genomic AvaII - EcoRI fragment is about 115 bp larger than its cDNA counterpart.

We conclude that the cysteine proteinase I gene is a single copy gene and the transcribed region is approximately 1500 bp in length. It contains at least two introns, one in the 5' 475 bp EcoRI - EcoRI fragment and the other in the 280 bp AvaII - EcoRI fragment.

2. CHROMATIN STRUCTURE OF THE CODING REGION OF THE CYSTEINE PROTEINASE I GENE

The cysteine proteinase I gene is developmentally regulated (Williams and Lloyd, 1979). We wished to examine changes in the chromatin structure of the cysteine proteinase I gene during development. The chromatin structure of the coding region was investigated by digestion of isolated nuclei with micrococcal nuclease and restriction endonucleases.

(a) Changes in cysteine proteinase I mRNA concentrations during development

Firstly, the developmental kinetics of the expression of the cysteine proteinase I gene were determined using Northern blot hybridization (Fig. 6a). Equal amounts (5 μ g) of total cellular RNA or poly(A⁺) RNA isolated from cells of the indicated developmental stages were size fractionated on 1.5% agarose gels, containing 6% formaldehyde. The RNA was transferred to DBM paper and hybridized with the 800 bp BglIII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1). Total cellular RNA from prespore and prestalk cells of the 15 h developmental stage was isolated after separation of the two cell types on Percoll density gradients (see Material and Methods). The mRNA of the cysteine proteinase I gene was present at a very low concentration in vegetative cells (Fig. 6a, lanes 1 and 11), 2 h and 4 h stages of development (lanes 2 and 3). A large increase in the level occurred after 6 h (Fig. 6a, lane 4), reached a maximum at the 8 h stage (lane 5) and decreased slowly after 12 h and 15 h (lanes 6 and 7). At the 15 h stage the level of cysteine proteinase I mRNA begins rapidly to fall (Bogdanovsky-Sequeval et al., 1984; Williams et al., 1985). The relatively high level remaining at 15 h presumably reflects the stability of the mRNA (see later). After aggregation, the average half-life of developmentally regulated mRNAs in D. discoideum is about 4 h (Chung et al., 1981;

Mangiarotti et al., 1982) As shown in Fig. 6a (lanes 8 and 9) the mRNA was found in prestalk and prespore cells at comparable concentrations.

(b) Micrococcal nuclease digestion

Micrococcal nuclease cuts preferentially in the DNA linker region between nucleosomes. Under conditions of mild digestion a ladder of DNA fragments is generated. In D. discoideum these fragments are on the average multiples of 180 bp (Parish et al., 1977).

We wished to determine if there is a change in the micrococcal nuclease pattern when the cysteine proteinase I gene becomes activated.

Nuclei were isolated from different stages of development and digested with micrococcal nuclease (Fig 7). Vegetative cells were digested with 80 units/ml of micrococcal nuclease, all differentiating stages were digested with 50 units/ml. Incubation times were 15, 30, 60, 120 and 300 s with (Fig. 7, lanes 3 to 7) or 300 s without micrococcal nuclease (Fig. 7 lanes 2). All digestions were performed at 25°C. Pure nuclear DNA was digested with 10 units/ml at 0°C (lane 1). The resulting DNA fragments were separated on a 2.3% agarose gel and stained with ethidium bromide. A series of bands was generated with a repeat of around 180 bp. The mononucleosomes gave two bands, the core size DNA (146 bp) and the chromatosome plus linker DNA (166-180 bp) which carries an H1-like protein (Parish and Schmidlin, 1985). The band pattern did not change during differentiation.

In Fig. 8, DNA fragments from similar gels to that shown in Fig. 7 were transferred to DBM paper and subsequently probed with the 800 bp BglIII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1). The typical nucleosome pattern was seen in vegetative and 4 h stage

cells (Fig. 8, veg and 4 h). The background was low and, even at the shortest digestion times, discrete bands were generated. However, at the 6 h stage a smearing of the pattern (ie. a continuous distribution of DNA fragments) was observed and reached a maximum at the 8 h stage (Fig. 8, 6 h and 8 h). Diffuse bands of the nucleosomal repeat were still apparent behind the smear. At the 12 h stage the smearing was reduced and by 15 h of development the vegetative cell pattern had reappeared (Fig. 8, 12 h). Pure nuclear DNA digested with micrococcal nuclease and probed with the cDNA of the cysteine proteinase I gene gave a smear (Fig. 8 lane 1). A comparison between the digestion kinetics of vegetative and 8 h stage cells show that the coding region of the cysteine proteinase I gene was not digested more quickly when transcribed. The sizes of the nucleosome bands were identical

(c) Sensitivity to digestion by restriction endonucleases

Ness et al. (1983) found the chromatin of the coding region of the D. discoideum ribosomal genes was only accessible to restriction enzymes when being transcribed. The sites in the nucleosome packaged non-transcribed spacer, however, were not cleaved. We wanted to ascertain whether the coding region of cysteine proteinase I gene becomes accessible to restriction endonucleases when the gene is turned on.

Nuclei of vegetative, 6 h, 8 h and 15 h stage cells were digested with 200 units EcoRI or HindIII for 30 min at 37°C. The DNA fragments were purified and the HindIII digested samples cut to completion with BglII. The resulting DNA fragments were size fractionated on a 1.5 % agarose gel, transferred to DBM paper and hybridized with the 800 bp BglII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig 1).

Fig. 9 shows the accessibility of the coding region of the cysteine proteinase I gene to EcoRI and HindIII during development. Both enzymes recognize a 6 bp sequence. Complete digestion of pure nuclear DNA with EcoRI resulted in a two bands, 475 bp and 440 bp in length, both originating from the coding region (see Fig. 4). The adjacent EcoRI fragments were large and hybridized weakly to the cDNA insert due to the short complementary sequences (Fig. 9, lane 1). When nuclei from the various developmental stages were digested with EcoRI (Fig. 9, lanes 2 to 5), the two EcoRI fragments originating from the coding region were only excised to a minimal extent, giving very weak hybridization signals. No change in the accessibility of the EcoRI sites in the coding region between the four developmental stages was seen (Fig. 9, lanes 2 to 5). A large number of additional fragments were obtained which represented star activity of EcoRI (Fig. 9, lanes 2 to 5). The star activity could not be inhibited even under high salt conditions (100 mM NaCl).

A double digest of pure nuclear DNA with HindIII and BglII generated a DNA fragment of 420 bp derived from the coding region. The adjacent fragments were more than 10 kb (Fig. 9, lane 6). Nuclei of the four developmental stages were treated with HindIII and the resulting DNA fragments cut to completion with BglII. The data in Fig. 9 (lanes 7 to 10) show that only a small fraction of the 420 bp BglII - HindIII fragment was released. Most of the cysteine proteinase I cDNA complementary sequences were seen at the top of the gel. No change in the accessibility of the HindIII site in the coding region was seen during development (Fig. 9, lanes 7 to 10). The additional band seen after HindIII digestion was due to endogenous nuclease activity (Fig. 9, lanes 7 to 10; see below).

Similar results were obtained with HinfI, which recognizes a 5 bp sequence (results not shown).

In summary, probing the chromatin with micrococcal nuclease showed the coding region of the inactive cysteine proteinase I gene was packaged into nucleosomes. A smear superimposed on diffuse nucleosome bands was seen when the gene was turned on. However, the kinetics of digestion did not change. When nuclei from various stages of development were digested with EcoRI, HindIII or HinfI, the sites within the coding region were considerably but not entirely protected throughout development. These results indicate that the chromatin structure of the coding region is altered during transcription but, unlike ribosomal genes the DNA is still protected against restriction enzyme attack.

3. CHROMATIN STRUCTURE OF THREE FURTHER GENES TRANSCRIBED BY RNA POLYMERASE II

As a control for the data obtained with the cysteine proteinase I gene, we examined the chromatin structure of three additional developmentally regulated genes.

- A gene that is constitutively expressed throughout development (clone pCZ22).
- A gene that is first transcribed during the late stages of development (clone pSC253).
- A gene that is transitorily active during the early stages of differentiation (clone pDd812).

(a) Clone pCZ22

pCZ22 is a cDNA clone complementary to a gene which is constitutively expressed throughout development (Zuker and Lodish, 1981).

Changes in pCZ22 mRNA concentration during development

Fig. 10a shows a Northern blot hybridization of pCZ22 to total cellular RNA from various stages of development, separated on a 1.5% agarose gel containing 6% formaldehyde and transferred to DBM paper. A high concentration of RNA complementary to pCZ22 was seen in vegetative cells (Fig. 10a, lane 1). In 4 h cells the level had decreased but then remained constant for the rest of development (Fig. 10a, lanes 2 to 6). pCZ22 RNA was detected in both prespore and prestalk cells isolated from the 15 h developmental stage (Fig. 10a, lanes 7 and 8).

Southern blot analysis

The gene was further characterized by Southern blot analysis (Fig. 11a). Nuclear DNA was digested with various restriction enzymes, the purified DNA fragments were separated on 1.5% agarose gels, transferred to DBM paper and hybridized with pCZ22. Digestion with all the restriction enzymes shown in Fig. 10a gave a number of bands complementary to pCZ22, indicating pCZ22 is a gene which exists in multiple copies per genome.

Micrococcal nuclease digestion

The chromatin structure of the coding region of pCZ22 was examined by probing with micrococcal nuclease. In Fig. 13 similar blots as shown in Fig. 7 were hybridized to pCZ22. Nuclei from various stages of development were digested with micrococcal nuclease (see Fig. 7), the purified DNA fragments were size fractionated on 2.3% agarose gels and transferred to DBM paper. In all the stages a smear between a weak nucleosome repeat was observed (Fig. 13, veg, 4 h, 8 h and 15 h). The extent of smearing did not change significantly during development. The sizes of the nucleosomal repeat were 180 bp and its multimers, identical to the ones seen after ethidium bromide staining (see Fig. 7).

Pure nuclear DNA digested with micrococcal nuclease and probed with pCZ22 showed a smear (Fig. 13, lane 1).

Hence, pCZ22 is complementary to a multiple copy gene constitutively expressed throughout development. The gene encodes for a single mRNA sequence. The chromatin structure of the coding region examined by micrococcal nuclease gave a smear superimposed on a faint nucleosome repeat. These results are similar to those obtained with the actively transcribed cysteine proteinase I gene.

(b) Clone pSC253

Zuker and Lodish (1981) and Chung et al. (1983) reported that the genomic clone pSC253 contains a 2700 bp EcoRI fragment of a single copy, developmentally regulated gene. Transcription commences after 15 h of differentiation.

Changes in pSC253 mRNA concentration during development

The data obtained by Northern blot hybridization of total cellular RNA isolated from various stages of development to pSC253 are presented in Fig. 10b. No mRNA complementary to pSC253 was detected in vegetative, 4 h, and 8 h stage cells (Fig 10b, lanes 1 to 3), whereas at the 12 h stage a faint band was seen (lane 4). After 15 h of development the level of this band strongly increased and several minor bands appeared (lane 5). After 18 h of development a smear was superimposed over these bands (lane 6). We identified a number of mRNAs in addition to the two described by Lodish and coworkers (Zuker and Lodish, 1981; Chung et al., 1983). Prespore and prestalk cells of the 15 h stage contained similar levels of pSC253 mRNA (Fig 10b, lanes 7 and 8).

Southern blot analysis

The analysis of the genomic DNA restriction fragments hybridizing to clone pSC253 (Fig. 11b) was performed with the Southern blot that had previously been probed with pCZ22 (Fig. 11a). After digestion with EcoRI, HindIII and KpnI (Fig. 11b, lanes E, H and K) only one band was observed, indicating that there were no cleavage sites for these enzymes within the coding region of pSC253. Digestion with BglII and HaeIII (lanes B and He) gave one major and two minor bands. HinfI (lane Hf) cut once in the coding region, resulting in two bands. These results confirm the data obtained by Chung et al. (1983), indicating pSC253 is a single copy gene.

Micrococcal nuclease digestion

Gels similar to that shown in Fig. 7 were blotted and hybridized with pSC253. Fig. 14 shows the micrococcal nuclease-generated DNA fragments isolated from nuclei digested at various developmental stages and hybridizing with pSC253. The classical nucleosomal repeat was seen in vegetative, 4 h, 8 h, 12 h and 15 h stage cells (Fig. 14, veg 4 h, 8 h, 12 h and 15 h). The background between the bands was low. At the 18 h stage, when the gene is first active, smearing between the bands became apparent (Fig. 14, 18 h). Due to the formation of spore and stalk cells, which produce cellulose-rich cell walls, nuclei could not be isolated from later stages when the gene becomes fully active. Like all other clones tested, the nucleosome repeat size in the coding region of pSC253 was about 180 bp and did not change during development (see Fig. 14). Pure nuclear DNA digested with micrococcal nuclease and hybridized with pSC253 showed a smear (Fig. 14, lane 1).

In summary pSC253, is a developmentally regulated, single copy gene which is first expressed during the late stages of development. The inactive gene is packaged into nucleosomes and the chromatin structure becomes more accessible to micrococcal nuclease digestion when transcription commences. We were unable to study the chromatin structure of the fully active gene since the cell wall formation at the late stages of development preclude the isolation of nuclei.

(c) Clone pDd812

pDd812 is a cDNA clone containing 570 bp of the discoidin Ia mRNA (Williams et al., 1979; Tsang et al., 1981). The discoidin I genes of D. discoideum (strain Ax2) constitute a family of three closely related, co-ordinately regulated genes (Williams et al., 1979; Tsang et al., 1981; Devine et al., 1982). By 6 h the discoidin I mRNA level has increased to about 1% of the total poly(A⁺) RNA (Ma and Firtel, 1978; Rowekamp et al., 1980; Devine et al., 1982). We used the 330 bp HindIII - EcoRI cDNA fragment of pDd812 for hybridization experiments (Williams et al., 1979; see Fig. 12).

Changes in discoidin I mRNA concentrations during development

The Northern blot which had previously been probed with pDdl1.7.10 (Fig 6a) was stripped and rehybridized with pDd812 (Fig. 6b) Low levels of mRNA complementary to pDd812 were present in vegetative cells (Fig. 6b, lane 1) and increased to a maximum at 6 h (lanes 2 to 4). Subsequently, transcription ceased and the mRNA was degraded. At the 8 h stage the concentration of mRNA had considerably dropped (Fig. 6b, lane 5) and in later stages no discoidin I mRNA was observed (lanes 6 and 7). The separated poly(A⁻) and the poly(A⁺) fractions of total cellular RNA from vegetative cells were probed with pDd812 (Fig. 6b, lanes 10 and 11).

All of the discoidin I mRNA was detected in the poly(A⁺) RNA fraction.

Southern blot analysis

The restriction maps of the three discoidin I genes in several strains of D. discoideum were determined in detail by Poole and Firtel (1984). In Fig. 10c a similar blot as shown in Fig. 10a and b was hybridized to the 330 bp HindIII - EcoRI fragment of pDd812. All the major restriction fragments seen in Fig. 10c coincide with the restriction map of the discoidin I genes in strain NC-4. However, after digestion with all the restriction enzymes shown in Fig. 10c, one or two additional bands were seen, giving a weak signal (see eg. lane H). They probably represent sequences of a pseudogene homologous with the discoidin I gene since such pseudogene fragments have been found in several strains of D. discoideum (Poole and Firtel, 1984).

Micrococcal nuclease digestion

Nuclei were isolated from various developmental stages, digested with micrococcal nuclease (see Fig. 7) and the DNA fragments hybridized with pDd812 (Fig. 15). In vegetative cells a nucleosome repeat and some smearing between the bands was seen (Fig. 15, veg). In the 4 h and 6 h stage the smearing was reduced and vanished totally after 15 h of development (Fig. 15, 4 h, 6 h, and 15 h). The size of the nucleosomal repeat did not change during development and was a multimer of 180 bp. Pure nuclear DNA cut with micrococcal nuclease and probed with pDd812 gave a continuous distribution of DNA fragments (Fig. 15, lane 1).

In summary, the discoidin I genes are active during early development. Although the discoidin I mRNA is as abundant as the cysteine proteinase I mRNA, (ie. the gene is transcribed at a high rate) only a slightly smeared nucleosomal pattern was seen even

when the discoidin gene family was fully active. These contradictory results are probably due to the variable expression of the discoidin I genes in the in the cells of early aggregates, some cells synthesizing little or no discoidin I (Barondes et al., 1983).

4. CHROMATIN STRUCTURE OF THE FLANKING REGIONS OF THE CYSTEINE PROTEINASE I GENE STUDIED BY INDIRECT END-LABELLING

The aim of these experiments was to investigate possible relationships between nuclease hypersensitive sites in the 5' and 3' flanking regions of the cysteine proteinase I gene and its regulation during development. Hypersensitive sites resulting from digestion with an endogenous nuclease and micrococcal nuclease were detected. These sites were mapped using the indirect end-labelling technique first applied by Nedospasov and Georgiev (1980) and by Wu (1980)

After mild digestion of nuclei with micrococcal nuclease or autodigestion by an endogenous nuclease, the purified DNA was cleaved with an appropriate restriction enzyme, size fractioned on agarose gels and transferred to nitrocellulose filters. Hybridization was performed with a short ^{32}P -labelled DNA fragment specific for one end of the chosen restriction cut within the genome. The fragments which extended from the chosen restriction site carrying the sequence complementary to the hybridization probe were visualized, the largest being the uncleaved restriction fragment. The size of the smaller fragments gave the position of the nuclease cleavage sites relative to the restriction site.

For the indirect end-labelling experiments we have used the 517 bp ScaI - BglII cDNA fragment of pDdl1.7.10 which has been earlier described (see Fig. 1). Southern blot analysis showed no

cross-hybridization with any other DNA sequence under the hybridization conditions used (see Fig. 2). All the indirect end-labelling experiments mapping in the 5' direction were repeated with a 209 bp HindIII - ScaI cDNA fragment of pDdl1.7.10 (see Fig. 1). Identical results were obtained but the hybridization signal was considerably weaker (results not shown).

To map the position of the hypersensitive sites in the 5' flanking region the DNA fragments were cleaved with AvaII. The AvaII site in the coding region lies 5 bp downstream from the ScaI site (see Fig. 4). To map in the 3' direction the DNA was cleaved with BglII (see Fig. 4). AvaII and BglII, which cut once in the coding region of the cysteine proteinase I gene (Fig. 4), both generate restriction fragments of at least 12 kb in the 5' and 3' flanking regions of the gene.

(a) Endogenous nuclease cleavage

We had previously observed endogenous nuclease activity in isolated nuclei. We wanted to examine whether the accessibility of the flanking regions of the cysteine proteinase I gene to the endogenous nuclease changed during development, ie. was related to gene regulation. Further, we wished to map the positions of the cleavage sites in the 5' and 3' flanking regions of the cysteine proteinase I gene using the indirect end-labelling technique described above.

Fig. 16 shows the endogenous nuclease sites in the 5' flanking region of the cysteine proteinase I gene at three developmental stages. Nuclei from vegetative, 8 h and 15 h stage cells were incubated on ice (Figs. 16 and 17, lanes 2, 4 and 6) or at 25°C for 20 min (Figs. 16 and 17, lanes 3, 5 and 7). The DNA was extracted and cut with AvaII or BglII for the 5' and 3' mapping respectively. The fragments were separated on 1.5% agarose gels,

transferred to nitrocellulose and hybridized with the BglII - ScaI cDNA fragment of pDdl1.7.10.

In vegetative (Fig. 16, lanes 2 and 3) and 15 h stage cells (lanes 6 and 7), when the gene was inactive, only weak endogenous nuclease cleavage was observed in the 5' region. At 8 h, however, when transcription was occurring, strong cleavage sites were observed 1350, 1430, 1800, 1900, 4300, 4700 and 6400 bp upstream from the AvaII site (Fig. 16, lanes 4 and 5). The start of the gene could not be precisely mapped but is approximately 1000 bp upstream from the AvaII site (see Fig. 5).

Endogenous nuclease cutting in the 3' downstream region was also barely detectable in vegetative (Fig. 17, lanes 2 and 3) and 15 h cells (lanes 6 and 7), with the exception of cleavages 6400 and 8000 bp downstream from the BglII site. In 8 h cells a number of cleavages were detected, the strongest being 1600, 1700 and 3100 bp downstream from the BglII site (Fig. 17, lanes 4 and 5). Cutting at the 8000 bp site was greatly increased. The end of the transcribed region is approximately 1250 bp downstream from the BglII site (see Fig. 5).

The maps of the endogenous nuclease cleavage sites in the 5' and 3' flanking region of the cysteine proteinase I gene are shown in Fig. 18 and Fig. 19 respectively.

In summary, specific sites upstream and downstream from the cysteine proteine I coding region were cleaved by an endogenous nuclease in nuclei isolated from the 8 h developmental stage, ie. when the gene was being transcribed. These data suggest that the endogenous nuclease was largely absent in the two other stages. Alternatively the chromatin in vegetative and 15 h stage cells may have an altered structure which prevents the endogenous nuclease cutting.

(b) Micrococcal nuclease digestion

We wanted to map the positions of micrococcal nuclease hypersensitive sites in the 5' and 3' flanking regions of the cysteine proteinase I gene at various stages of development. Nuclei from vegetative cells, 8 h and 15 h stages of development were digested with 12.5 units/ml micrococcal nuclease at 25°C. Incubation times were 30, 60 and 120 s (Figs. 20 and 21, lanes 3 to 7) with or 300 s (Figs. 20 and 21, lane 2) without micrococcal nuclease. Pure DNA was digested with 10 units/ml micrococcal nuclease at 0°C (Figs. 20 and 21, lane 1). The DNA was extracted from nuclei and cut with *Ava*II or *Bgl*II (see above). The resulting DNA fragments were size fractionated on 1.5% agarose gels, transferred to nitrocellulose filters and hybridized with the *Bgl*II - *Sca*I cDNA fragment of pDdl1.7.10.

The hypersensitive sites in the 5' upstream region of the gene are shown in Fig. 20. Micrococcal nuclease displays a strong DNA sequence specificity (Dingwall et al., 1981; Hörz and Altenburger, 1981). However, only two of the cleavage sites obtained following digestion of naked DNA (Fig. 20, lane 1) were also present in the nuclear chromatin digests. These were 540 bp and 950 bp upstream from the *Ava*II site and therefore both within the coding sequence. A dramatic change in the pattern of hypersensitive sites was observed between vegetative (Fig. 20, veg) and 8 h cells (Fig. 20, 8 h). Many of the sites in vegetative cells were absent from 8 h cells and the majority of the new sites corresponded to regions cut by endogenous nuclease. Most prominent were the two double bands 1350/1430 bp and 1800/1900 bp upstream from the *Ava*II site (Fig. 20, 8 h). A strong band appeared at 4700 bp. The 4300 bp site cleaved by endogenous nuclease in 8 h stage cells (Fig. 20, 8 h, lane 2) was recognized by micrococcal nuclease at all stages of development (Fig. 20, veg, 8 h and 15 h). Hence, it is presumably always accessible. A change also occurred in the coding region, an

820 bp band appearing in 8 h cells (Fig. 20, 8 h). This band may reflect the loss of or a structural change in nucleosomes from the coding region. The nuclei from 15 h cells (Fig. 20, 15 h) gave a pattern almost identical to 8 h cells (Fig. 20, 8 h) except that the 1800/1900 bp double band had been replaced by an 1850 bp band. Cutting within the coding region resembled vegetative cells, the 700 bp and 760 bp band (Fig. 20, veg and 15 h) reappearing.

The changes in hypersensitive sites in the 3' flanking region of the gene resulting from transcription were only minimal (Fig. 21). Several endogenous nuclease cleavage sites, which appeared in 8h cells (Fig. 21, 8 h) but not in vegetative and 15 h cells (Fig. 21, 15 h), were accessible to micrococcal nuclease in the two latter stages. Thus, in vegetative cells (Fig. 21, veg) the sites at 1050 bp, 1700 bp and 2200 bp were recognized, whereas in the 15 h developmental stage (Fig. 21, 15 h) these sites were also cleaved along with sites at 2000 bp, 2400 bp and 3100 bp. In 8h cells (Fig. 21, 8 h), 4200 bp and 1600 bp bands typical of endogenous nuclease cleavage disappeared following longer micrococcal nuclease digestion times. They may be rapidly trimmed by the latter enzyme. The sites on naked DNA preferentially cut by micrococcal nuclease (Fig. 21, lane 1) did not coincide with sites in chromatin, the exception being a site 800 bp from the BglII cleavage site.

Maps of the micrococcal sensitive sites in the 5' and 3' flanking regions of the cysteine proteinase I gene are shown in Fig. 22 and Fig. 23 respectively.

Hence, in the 5' flanking region of the cysteine proteinase I gene a dramatic change occurred in the arrangement of micrococcal nuclease hypersensitive sites when the gene was turned on. However, the pattern was not significantly changed at the 15 h stage of development when transcription had largely ceased. Most of the

endogenous nuclease accessible sites were also recognized by the micrococcal nuclease.

No significant change was seen in the pattern of micrococcal nuclease hypersensitive sites in the 3' flanking region of the gene during development.

5. ARE THE FLANKING REGIONS OF THE CYSTEINE PROTEINASE I GENE CLEAVED BY TOPOISOMERASES?

The aim of these experiments was to identify possible topoisomerase cleavage sites in the 5' flanking region of the cysteine proteinase I gene. Treatment of diluted nucleoli of D.discoideum (Ness et al., in preparation) and Tetrahymena (Gocke et al., 1983) with high concentrations (1%) of sodium dodecyl sulphate induces topoisomerase activity which then cleaves the flanking regions of the ribosomal genes. In the 5' flanking region of D.discoideum ribosomal genes double strand cuts were induced, whereas in Tetrahymena only single strand cleavage sites were observed (Gocke et al., 1983).

The indirect end-labelling technique was used to detect and localize topoisomerase cutting sites in the 5' flanking region of cysteine proteinase I (Fig. 24). Nuclei were treated with either sodium dodecyl sulphate (Fig. 24a and b, lanes 3, 5 and 7) or NaCl (Fig. 24a and b, lanes 2, 4 and 6). The purified DNA was cleaved with AvaII, size fractionated on 1.5 % agarose gels (non- denaturing, Fig. 24a, and denaturing gels, Fig. 24b) and transferred to a nitrocellulose filter. Hybridization was performed with the 517 bp BglII - ScaI cDNA fragment. No sodium dodecyl sulphate inducible topoisomerase cutting sites were observed, either following separation on non-denaturing (Fig. 24a) or on denaturing gels (Fig. 24b).

All the bands detected (Fig. 24a and b) are due to the endogenous nuclease activity and coincide with the cleavage sites shown in Fig. 16.

In contrast to the ribosomal genes in D. discoideum no sodium dodecyl sulphate inducible topoisomerase cleavage sites were detected in the 5' flanking region of the cysteine proteinase I gene.

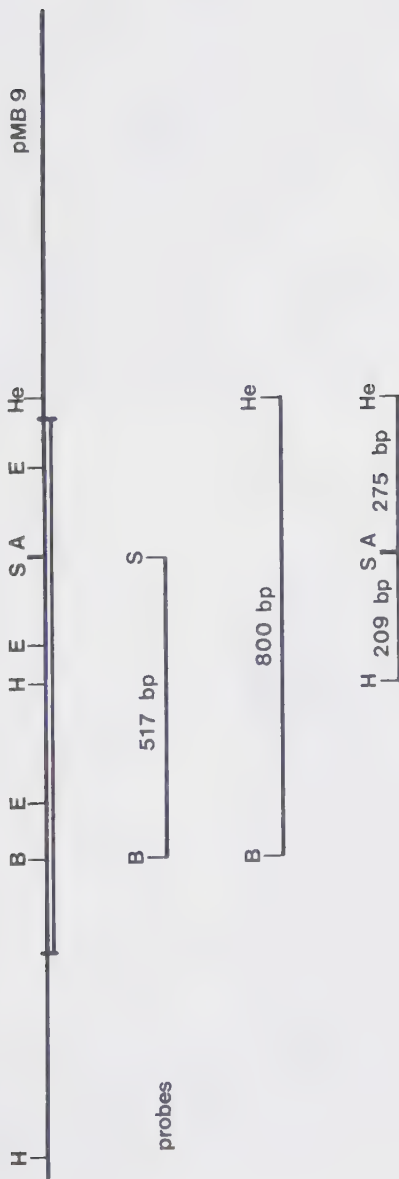


Fig. 1: pDd11.7.10, cDNA clone of the cysteine proteinase I gene. The cDNA fragments were isolated by digestion of the plasmid DNA with the appropriate restriction enzyme followed by fragment separation on 2% agarose gels. Individual fragments were intercepted using DEAE cellulose membrane and eluted. The symbols of the restriction enzymes used are: A, *Ava*I; B, *Bgl*II; E, *Eco*RI; H, *Hind*III; He, *Hae*III; S, *Sca*I.

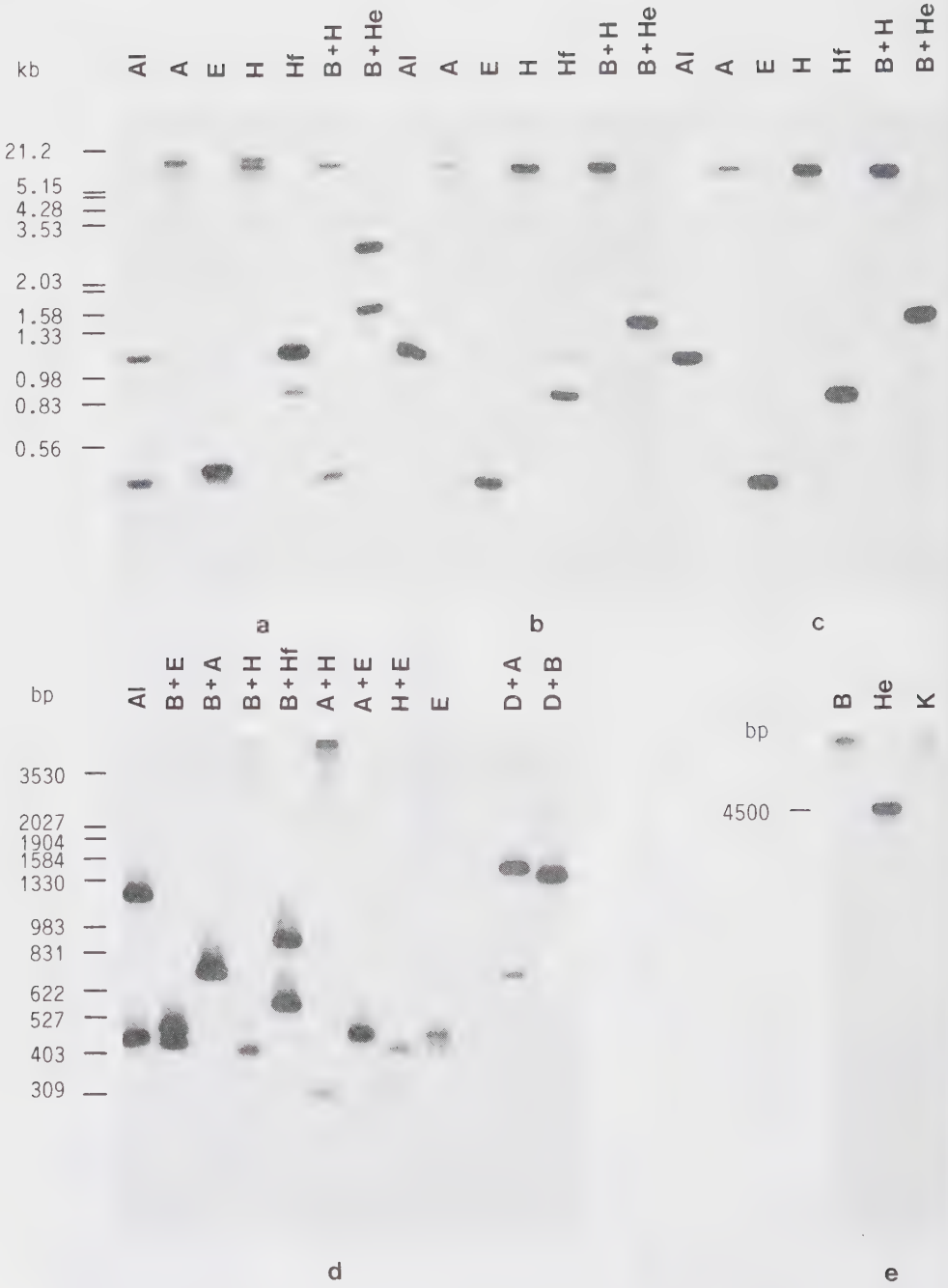


Fig. 2:

Fig. 2: Analysis of the genomic DNA restriction fragments hybridizing to cysteine proteinase I. Nuclear D. discoideum DNA was digested with the indicated restriction endonucleases, size fractionated on 1.5% (a, b, c and e) or 2.3% agarose gels (d), transferred to DBM paper and hybridized (a) with the 517 bp BglII - ScaI cDNA fragment, (b) with the 209 bp HindIII - ScaI cDNA fragment, (c) with the 275 bp AvaII - HaeIII cDNA fragment (d) and (e) with the 800 bp BglII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1). The symbols for the restriction enzymes are: A, AvaII; Al, AluI; B, BglII; D, DdeI; E, EcoRI; H, HindIII; He, HaeIII; Hf, HinfI; K, KpnI.

Subclone pSH 209

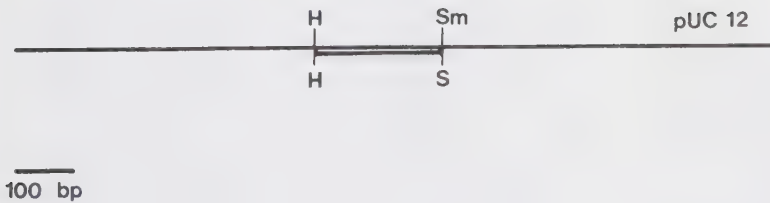


Fig. 3: Subclone SH209. The 209 bp HindIII - ScaI cDNA fragment of pDdl1.7.10 was subcloned using pUC12 as a vector. The symbols of the restriction enzymes used are: H, HindIII; S, ScaI; Sm, SmaI.

Cysteine proteinase I

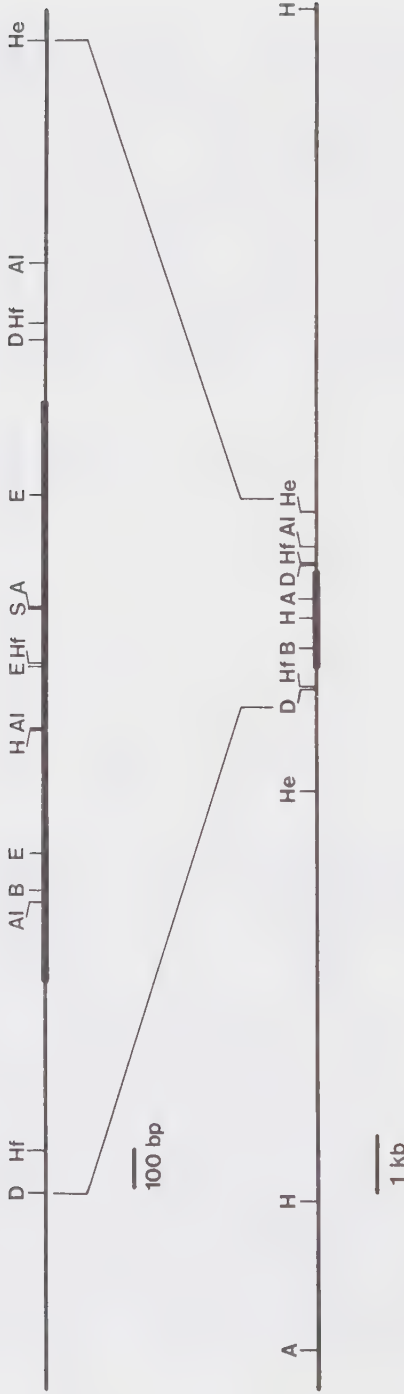


Fig. 4: Genomic map of the cysteine proteinase I gene. The symbols of the restriction sites are: A, AvaII; Al, AluI; B, BglII; D, DdeI; E, EcoRI; H, HindIII; He, HaeIII; Hf, HinfI; S, ScaI.

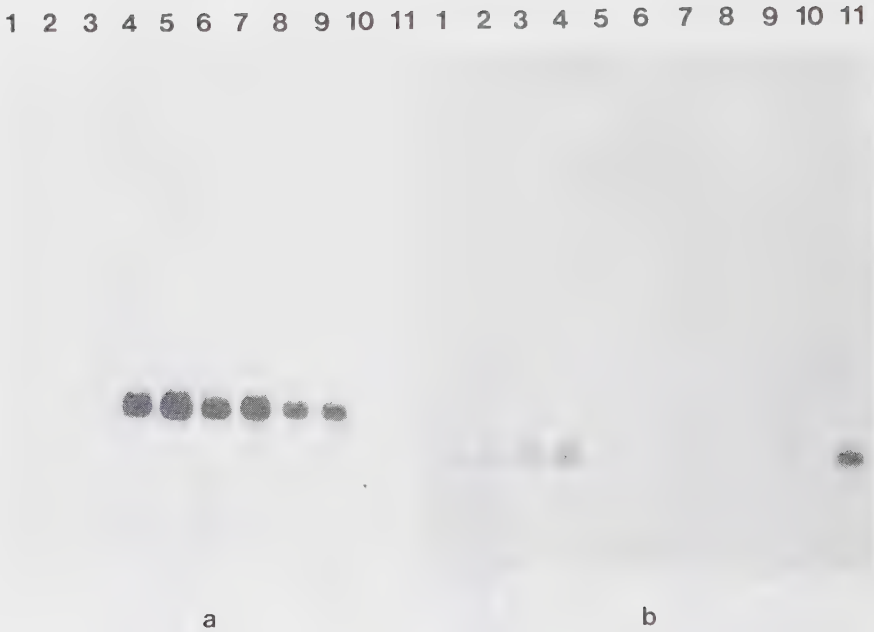


Fig. 6: Northern blot hybridization of (a) cysteine proteinase I mRNA and (b) discoidin I mRNA at various stages of the cell cycle. Lanes 1 to 7: Total cell RNA isolated from vegetative cells, 2 h, 4 h, 6 h, 8 h, 12 h and 15 h stages of development. Lanes 8 and 9: Total cell RNA from prespore and prestalk cells (15 h differentiation stage). Lanes 10 and 11: Poly A⁻ RNA and poly A⁺ RNA from vegetative cells. On each lane 5 μ g RNA was separated on a 1.5% agarose gel containing 6% formaldehyde, transferred to DBM paper and hybridized (a) with the 800 bp BglII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1), (b) with the 330 bp HindIII - EcoRI cDNA fragment of pDd812 (see Fig. 12).

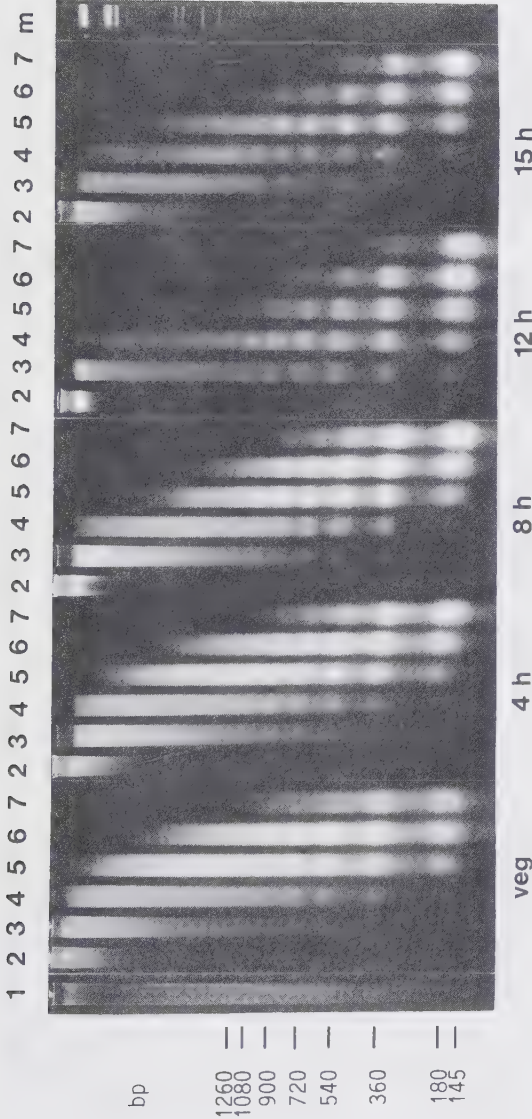


Fig. 7: Micrococcal nuclease digest of Dictyostelium nuclei. Isolated nuclei of the various stages indicated were incubated at 25°C with 80 units/ml (vegetative cells) or 50 units/ml micrococcal nuclease (differentiating stages). Incubation times were 15, 30, 60, 120 and 300 s with (lanes 3 to 7) or 300 s without micrococcal nuclease (lane 2). Pure nuclear DNA was digested with 10 units/ml at 0°C (lane 1). The purified DNA fragments were separated on a 2.3% agarose gel. λ Marker (lane m): DNA double-digested with EcoRI and HindIII.

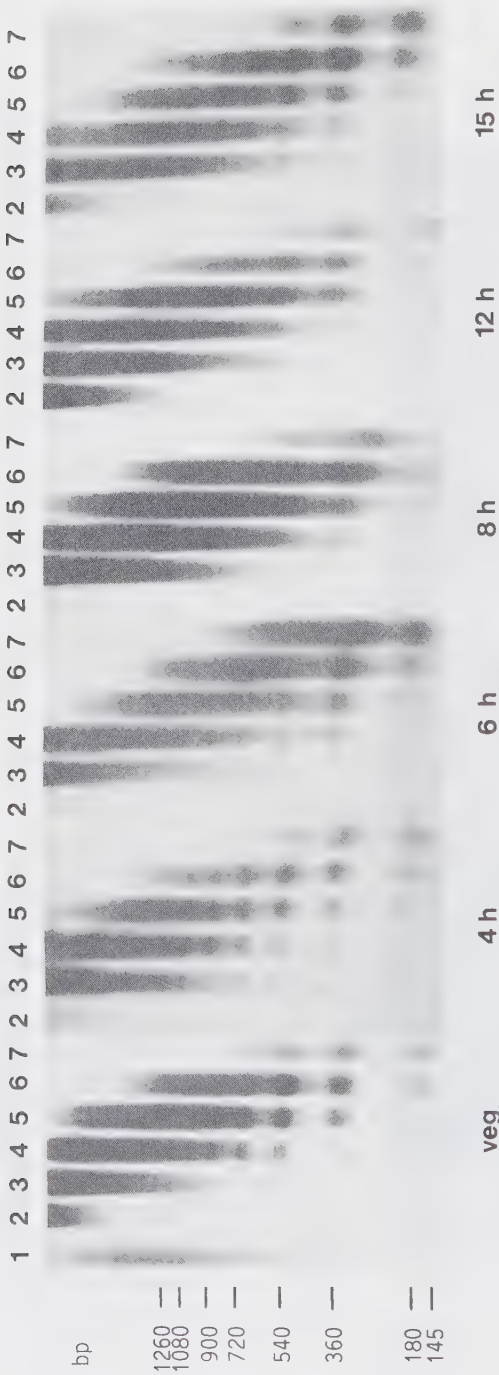


Fig. 8: Chromatin structure of the cysteine proteinase I gene during differentiation. Isolated nuclei from vegetative cells, 4 h, 6 h, 8 h, 12 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 7; digested pure DNA is in lane 1. Purified DNA was separated on 2.3% agarose gels, transferred to DBM paper and hybridized with the 800 bp BglII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1).

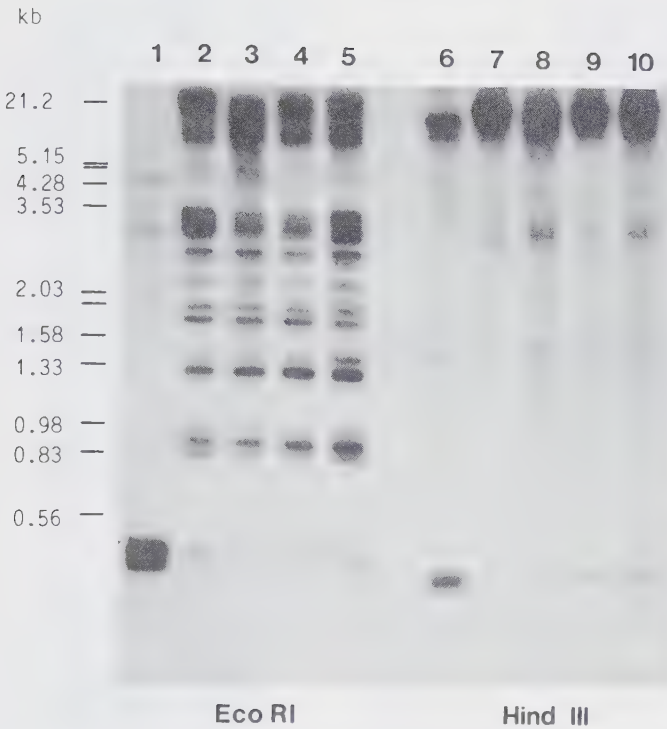


Fig. 9: Sensitivity of cysteine proteinase I chromatin to restriction endonuclease digestion. Nuclei from vegetative, 6 h, 8 h and 15 h differentiated cells were incubated for 30 min with EcoRI (lanes 2 to 5) or with HindIII (lanes 7 to 10) at 37°C. The DNA fragments were purified and the HindIII digested samples cut with BglII. Lane 1 shows pure nuclear DNA cut with EcoRI, lane 6, pure nuclear DNA cut with HindIII and BglII. The DNA fragments were size fractionated on a 1.5% agarose gel, blotted, and hybridized with the 800 bp BglII - HaeIII cDNA fragment of pDdl1.7.10 (see Fig. 1).

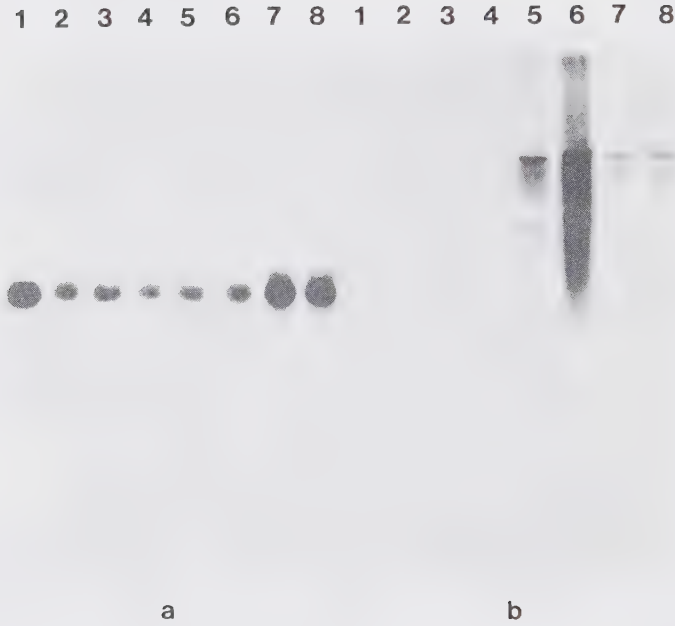


Fig. 10: Northern blot hybridization of mRNA hybridizing to (a) pCZ22 DNA and (b) pSC253 DNA at various stages of the cell cycle. Lanes 1 to 6: Total cell RNA isolated from vegetative, 4 h, 8 h, 12 h, 15 h, and 18 h differentiated cells. Lanes 7 and 8: Total cell RNA from prespore and prestalk cells (15 h differentiation stage). Separated and transferred RNA was hybridized (a) with pCZ22 DNA, (b) with pSC253 DNA.

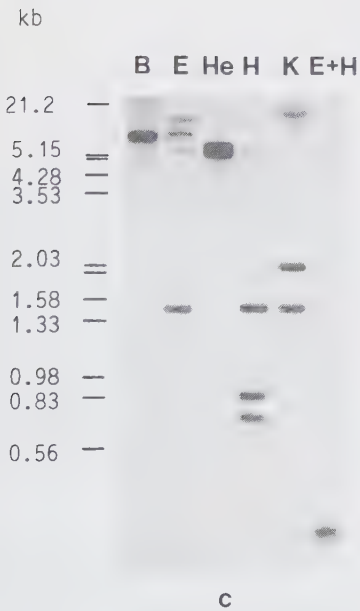
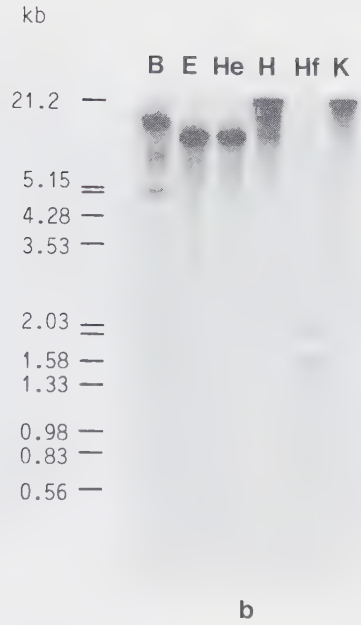
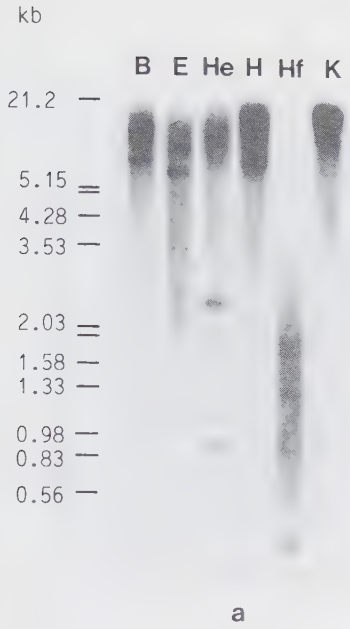


Fig. 11:

Fig. 11: Analysis of the genomic DNA restriction fragments hybridizing to pCZ22 (a), pSC253 (b) and pDd812 (c). Nuclear D. discoideum DNA was digested with the indicated restriction endonucleases, size fractionated on 1.5% agarose gels, transferred to DBM paper and hybridized (a) with pCZ22, (b) with pSC253 and (c) with the 330 bp HindIII - EcoRI cDNA fragment of pDd812 (see Fig. 12). The symbols for the restriction enzymes are: B, BglII; E, EcoRI; H, HindIII; He, HaeIII; Hf, HinfI; K, KpnI.

pDd 812



100 bp

Fig. 12: pDd812, cDNA clone of the discoidin I gene. The HindIII - EcoRI fragment was isolated as described in Fig. 1. The symbols of the restriction enzymes used are: E, EcoRI; H, HindIII.

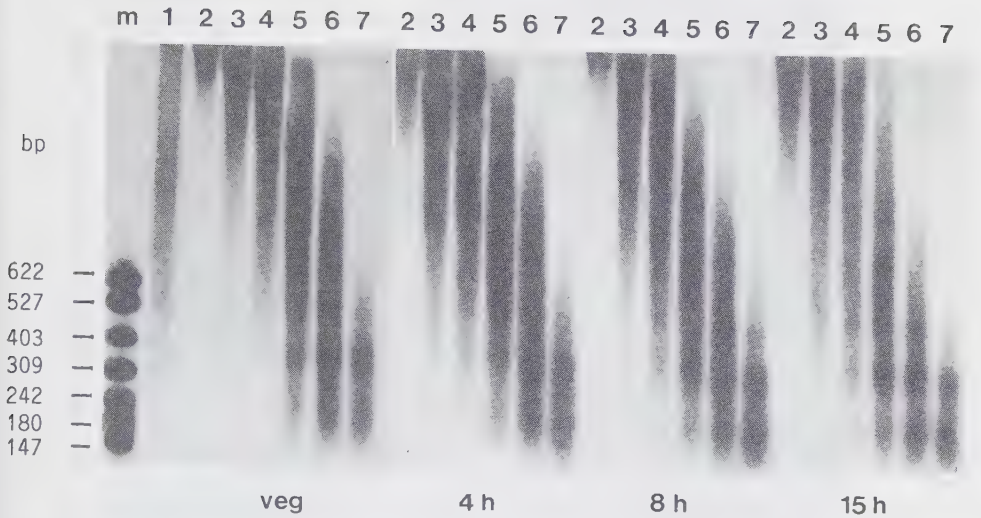


Fig. 13: Chromatin structure of the gene hybridizing to pCZ22 during differentiation. Isolated nuclei from vegetative cells, 4 h, 8 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 7; digested pure DNA is in lane 1. Purified DNA was separated on 2.3% agarose gels, transferred to DBM paper and hybridized with pCZ22. Marker (lane m): pBR322 DNA cut with HpaII.

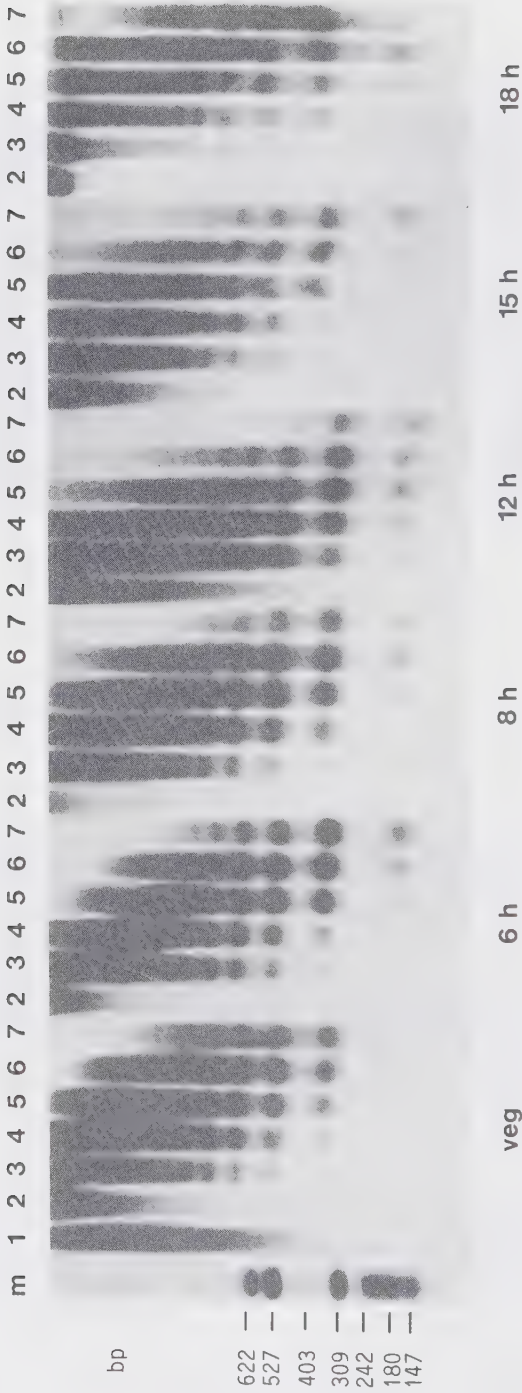


Fig. 14: Chromatin structure of the gene hybridizing to pSC253 during differentiation. Isolated nuclei from vegetative cells, 6 h, 8 h, 12 h, 15 h and 18 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 7; digested pure DNA is in lane 1. Purified DNA was separated on 2.3% agarose gels, transferred to DBM paper and hybridized with pSC253. Marker (lane m): pBR322 DNA cut with HpaII.

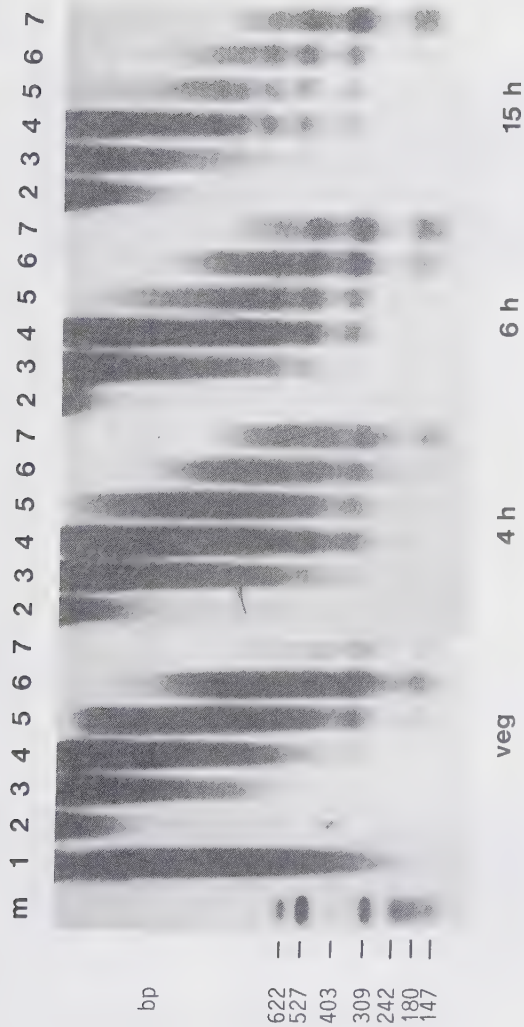


Fig. 15: Chromatin structure of the discoidin I gene during differentiation. Isolated nuclei from vegetative cells, 4 h, 6 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 7; digested pure DNA is in lane 1. Purified DNA was separated on 2.3% agarose gels, transferred to DBM paper and hybridized with the 330bp HindIII - EcoRI cDNA fragment of pDd12 (see Fig. 12). Marker (lane m): pBR322 DNA cut with HpaII.

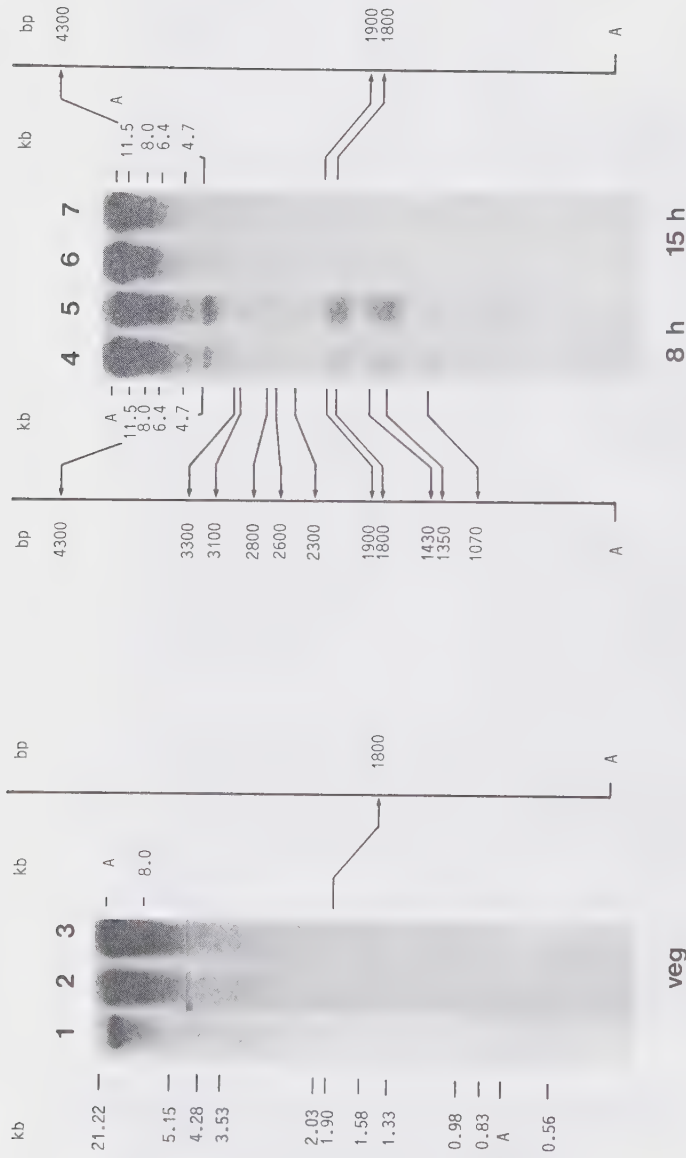


Fig. 16: Endogenous nuclease sites in the 5' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative cells, 8 h and 15 h differentiated stages were incubated for 20 min at 25°C (lanes 3, 5 and 7) and at 0°C (lanes 2, 4 and 6). Lane 1: total *D. discoideum* DNA digested with *Ava*II. The purified DNA fragments were cut with *Ava*II, separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the 517 bp *Bgl*II - *Sca*I cDNA fragment (see Fig. 1).

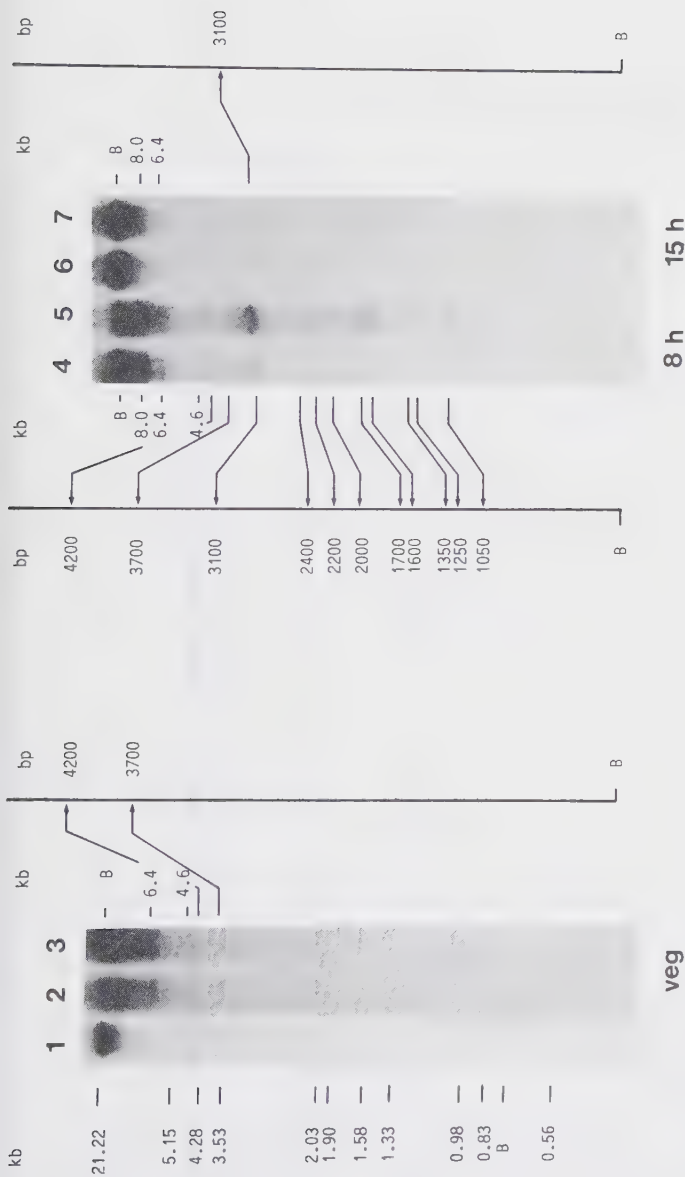


Fig. 17: Endogenous nuclease sites in the 3' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative cells, 8 h and 15 h differentiated stages were incubated for 20 min at 25°C (lanes 3, 5 and 7) and at 0°C (lanes 2, 4 and 6). Lane 1: total *D. discoideum* DNA digested with BglII. The purified DNA fragments were cut with BglII, separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the 517 bp BglII - ScaI fragment (see Fig. 1).



Fig. 18: Map of the endogenous nuclease cleavage sites in the 5' flanking region of the cysteine proteinase I gene at the three stages of development indicated (see Fig. 16).



Fig. 19: Map of the endogenous nuclease cleavage sites in the 3' flanking region of the cysteine proteinase I gene at the three stages of development indicated (see Fig. 17).

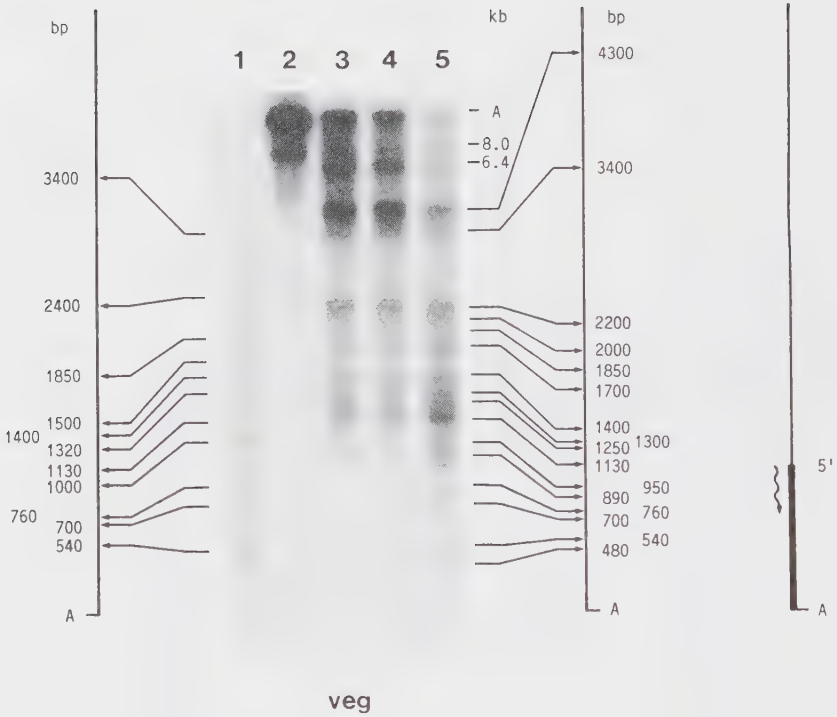


Fig. 20: Micrococcal nuclease hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative, 8 h and 15 h differentiated cells were digested with 12.5 units/ml micrococcal nuclease at 25°C. Incubation time was 30, 60 and 120 s with (lanes 3 to 5) and 300 s without micrococcal nuclease (lane 2). Pure nuclear DNA was digested with 10 units/ml at 0°C (lane 1). The purified DNA fragments were cut with *Ava*II, separated on a 1,5% agarose gel and transferred to a nitrocellulose filter. Hybridization was performed with the 517 bp *Bgl*III - *Sca*I cDNA fragment of pDdl1.7.10 (see Fig. 1).

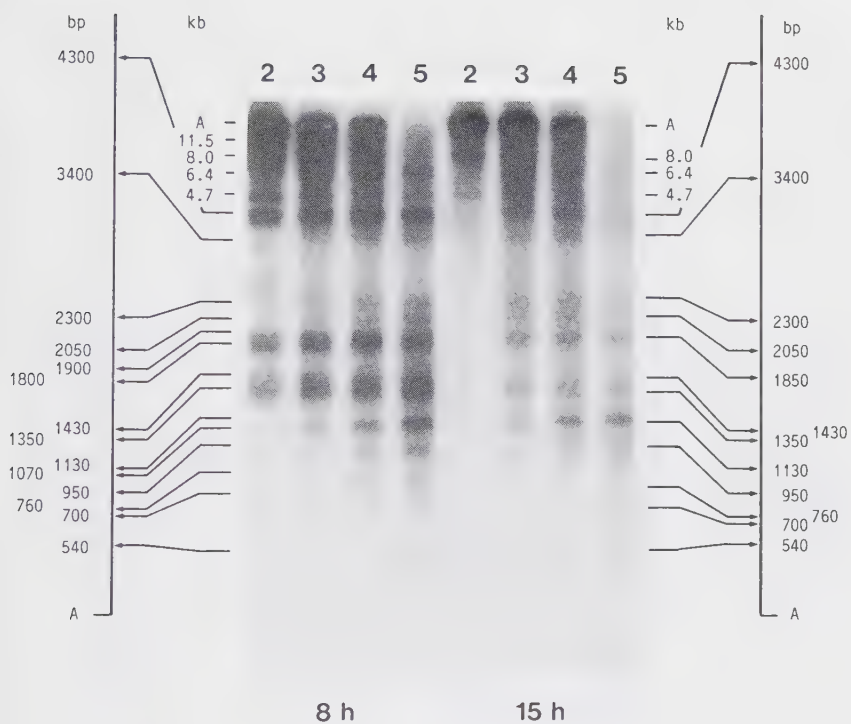


Fig. 20.

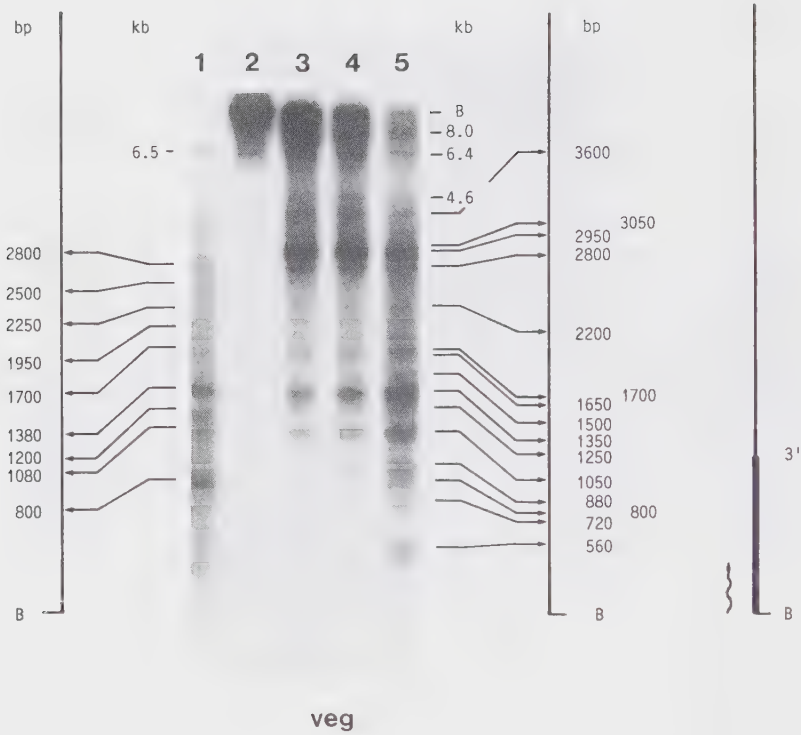


Fig. 21: Micrococcal nuclease hypersensitive sites in the 3' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative, 8 h and 15 h differentiated cells and pure nuclear DNA were digested with micrococcal nuclease (see Fig. 20). The purified DNA fragments were cut with BglII, separated on a 1,5% agarose gel and transferred to a nitrocellulose filter. Hybridization was performed with the 517 bp BglII - ScaI cDNA fragment (see Fig. 1).

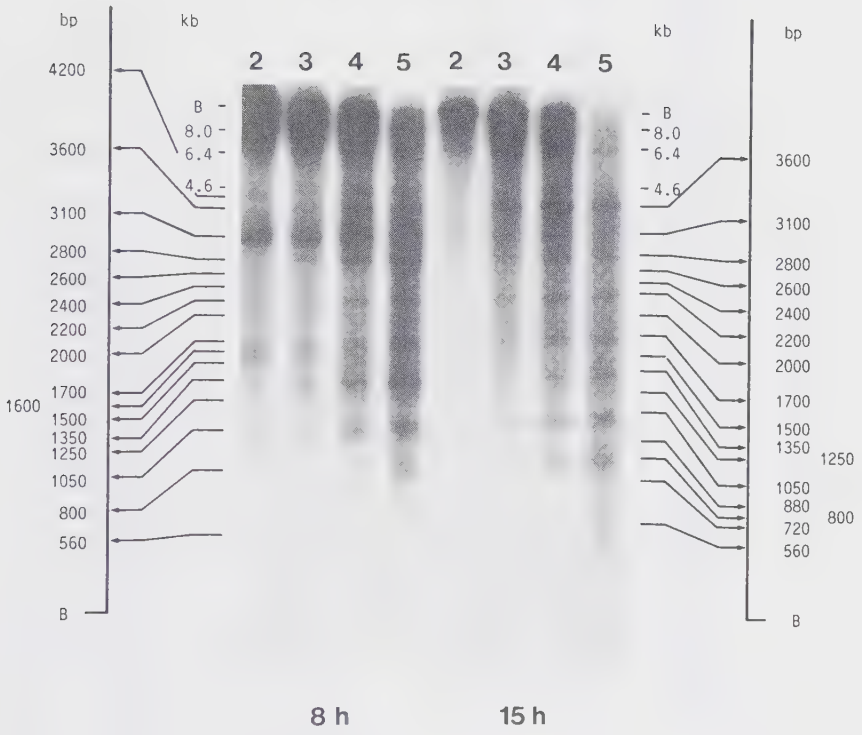


Fig. 21.



Fig. 22: Map of the micrococcal nuclease hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene in vegetative cells, 8 h and 15 h differentiation stages (see Fig. 20).



Fig. 23: Map of the micrococcal nuclease hypersensitive sites in the 3' flanking region of the cysteine proteinase I gene in vegetative cells, 8 h and 15 h differentiation stages (see Fig. 21).

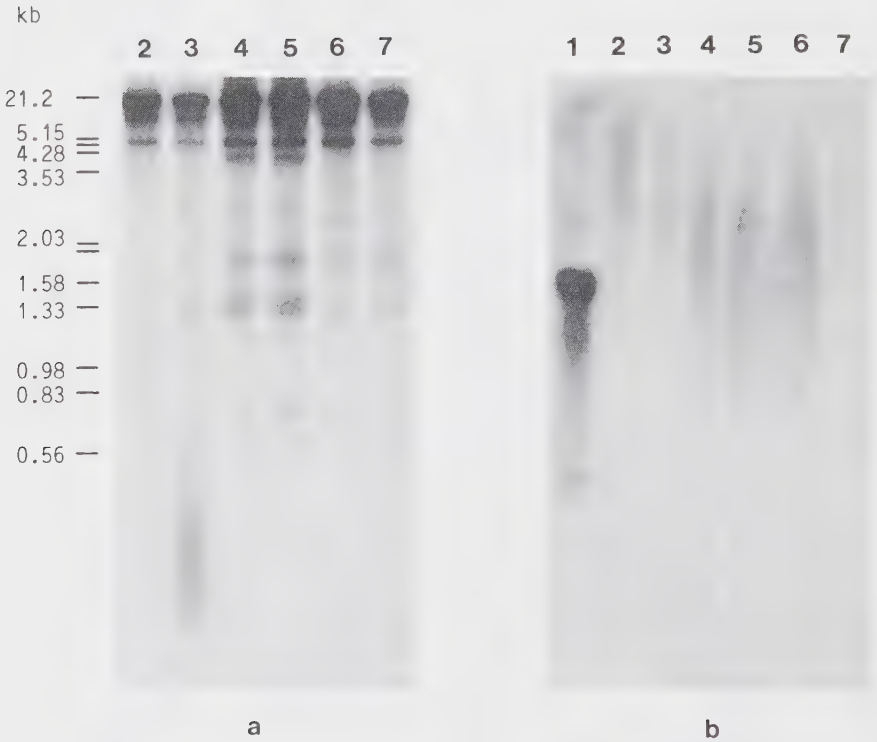


Fig. 24: Topoisomerase assay for the 5' flanking region of cysteine proteinase I. Nuclei from vegetative, 8 h and 15 h differentiated cells were treated with NaCl (lanes 2, 4 and 6) or sodium dodecyl sulphate (lanes 3, 5 and 7). Lane 1: Nuclear DNA digested with DdeI and AvaII. The purified DNA fragments were separated on a 1.5% agarose gel (a) or on a 1.5% agarose denaturing gel containing 30 mM NaOH (b). The size fractionated DNA fragments were blotted onto nitrocellulose filters and hybridized with the 517 bp BglII - ScaI cDNA fragment (see Fig. 1).

IV. DISCUSSION

1. MICROCOCCAL NUCLEASE DIGESTION OF CODING REGIONS

Analysis of the cysteine proteinase I gene by micrococcal nuclease digestion and blot hybridization showed that the coding region is in regularly repeating units (nucleosomes) when the gene is inactive. Following activation a continuous distribution of DNA fragments was observed. This lack of periodicity, which was seen as smear in electrophoresis, may result from an undefined linker length between the nucleosomes, loss of the nucleosomes or a significant change in their structure (Ness et al., 1983; Widmer et al., 1984). Although the nucleosome repeat could still be seen behind the smear, the smear did extend below the mononucleosomal size. The smear was not observed in another single copy gene (clone pSC253) which is inactive for most of development. On the other hand, clone pCZ22, which occurs in multiple copies and remains constitutively active, resembles cysteine proteinase I in giving a smear superimposed over a nucleosomal repeat.

We interpret the results in terms of a model in which the transcription complex carries the machinery required to open the nucleosome structure. When the density of transcription complexes is high, most of the nucleosomes will be in the "active" configuration and susceptible to micrococcal nuclease digestion. When the density falls, "normal" nucleosomes may reform before the next transcription complex comes along. Hence, nucleosomes and transcription complexes may occur together on the same coding sequence. Psoralen cross-linking experiments with ribosomal genes

of D. discoideum support this model (Sogo et al., 1984).

The results with the clone pDd812, using a fragment containing 330 bp from the 5' end of discoidin I cDNA, are relevant to the controversy concerning the absence or presence of nucleosomes on transcribing chromatin. Although the gene is apparently very active, mRNA rising to about 1% of the total cell poly(A⁺) RNA by six hours (Ma and Firtel, 1978; Rowekamp et al., 1980, Devine et al., 1982), only a faint smearing of the nucleosome pattern was observed following micrococcal nuclease digestion. The three discoidin I genes are highly conserved in the protein coding region and are under co-ordinate control (Poole et al., 1981; Devine et al., 1982). Nevertheless, the contradictory results could be explained if the genes are transcribed for only a short period and transcription is not synchronous in all cells. Moreover, immunohistochemistry indicated the cells had variable amounts of antigen and some cells may never express much discoidin I (Barondes et al., 1983).

2. ACCESSIBILITY OF THE CODING REGION OF CYSTEINE PROTEINASE I TO RESTRICTION ENDONUCLEASES

Sites within the coding region of actively transcribed ribosomal genes in D. discoideum were accessible to restriction enzymes, whereas sites within the non-transcribed spacer region were not (Ness et al., 1983). In 15 h cells, where the rate of ribosomal RNA synthesis is low, the accessibility of the coding region to restriction enzymes was much reduced. This reduction coincided with the return of a typical nucleosome repeat (Ness et al., 1983). A similar result was obtained with the hyperactive secretory protein gene of Chironomus, where a restriction enzyme with a 6 bp recognition sequence cut the gene in the hyperactive chromatin state but not in the inactive configuration (Widmer et al., 1984). Restriction enzymes recognizing 4 bp could not

distinguish between active and inactive chromatin, possibly because they can cut DNA wrapped around the nucleosome. However, we were unable to find any increase in the accessibility of the cysteine proteinase I gene to restriction enzymes when it was being transcribed. Similarly, Weischet et al. (1983) found BspR1 restriction sites within the active mouse kappa immunoglobulin gene appeared well protected even against a high excess of enzyme and although micrococcal nuclease digestion gave a smeared pattern of DNA fragments. The active coding region of D. discoideum ribosomal genes is more susceptible to micrococcal nuclease digestion than the inactive, nucleosome-containing gene (Ness et al., 1983). This was not so with cysteine proteinase I. Hence, the polymerase II transcription complex may protect DNA from endonuclease digestion more efficiently than the polymerase I transcription complex. However, the disagreement with the Chironomus results is puzzling.

3. ENDOGENOUS NUCLEASE AND MICROCOCCAL NUCLEASE HYPERSENSITIVE SITES FLANKING THE CYSTEINE PROTEINASE I GENE

The indirect end-labelling experiments showed that specific sites upstream and downstream from the cysteine proteinase I coding region were cleaved by an endogenous nuclease in 8 h cells, ie. when the gene was being transcribed (Fig. 16 - 19). These sites were also accessible for micrococcal nuclease in 15 h cells, indicating endogenous nuclease activity was largely absent at this stage. This was presumably also the case in vegetative cells since, although the majority of micrococcal nuclease hypersensitive sites upstream from the coding region differed from 8 h and 15 h sites, most of the downstream and some distant upstream sites were also cleaved by endogenous nuclease at the 8 h stage. Hence although the function of the endogenous nuclease is unknown, it becomes associated with the transcribed gene. Vegetative and 15 h cells do contain an

endogenous nuclease which cut specific regions upstream and downstream of the ribosomal DNA coding region (Ness et al., in preparation). The nucleoli of D.discoideum (Ness et al., in preparation) and Tetrahymena (Gocke et al., 1983) contain topoisomerase activity that also cleaves the 5' flanking region of the ribosomal genes. We have as yet been unable to demonstrate topoisomerase cleavage associated with the cysteine proteinase I gene.

The micrococcal nuclease cleavage sites in the 3' flanking region did not change significantly during development. However, dramatic differences between the repressed (vegetative cells) and transcribing (8 h cell) stage in the 5' flanking region were revealed. The pattern of hypersensitive sites did not revert to the repressed state when the gene was no longer transcribed in 15 h cells, so they were not a consequence of transcription.

4. A MODEL OF CYSTEINE PROTEINASE I GENE REGULATION

A model relating the changes in chromatin structure of the Dictysin I gene to gene activation and transcription is shown in Fig. 25. The exact initiation and termination points are not known and may be up to 100 bp longer than shown. The hypersensitive sites more than 1300 bp upstream or 2000 bp downstream are not shown. These sites may be associated with adjacent genes and since the site 3700 bp upstream becomes accessible in 8 h cells, it may be indicative of a gene coordinately regulated with the cysteine proteinase I.

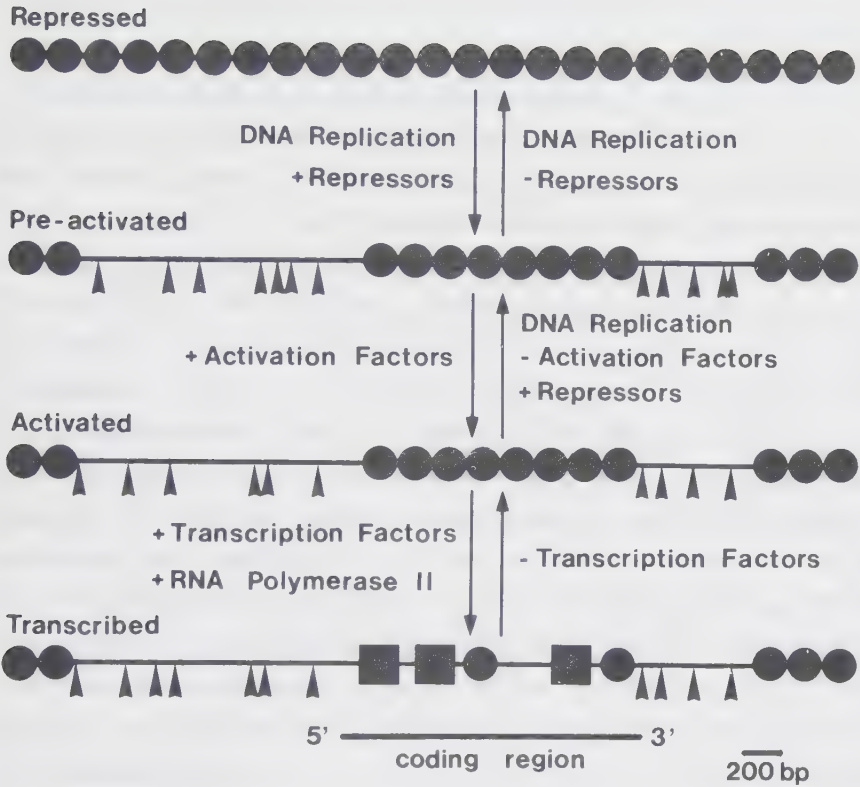


Fig. 25: Model of the changes in chromatin structure of the cysteine proteinase I gene during activation and transcription (see text for details). Black circles represent nucleosomes; arrowheads indicate micrococcal nuclease hypersensitive sites; black squares are transcription complexes. Although they may first bind upstream of the initiation site, transcription complexes are only shown associated with the coding region where they transitorily open the nucleosome structure. The repressed supranucleosomal state probably does not occur in the cysteine proteinase I gene.

The repressed gene and its flanking regions are shown as being completely packed into nucleosomes and presumably possess a supranucleosomal organization (Weintraub, 1984). However, the cysteine proteinase I gene probably never occurs in this state, the inactive gene being in the pre-activated state. The coding region of the pre-activated gene is packed into nucleosomes, although we cannot yet say whether these are phased. Since the double monomer band was detected, these nucleosomes carry the H1-like, lysine-rich protein (Parish and Schmidlin, 1985). However, it would be bound differently to the H1 associated with the coding region of repressed genes of higher organisms. The latter crosslinks adjacent nucleosomes and hence participates in supranucleosomal structure (Weintraub, 1984). Micrococcal nuclease will preferentially cleave the linker DNA between nucleosomes, however the distances between the hypersensitive sites in the regions flanking the gene are variable. Many of these sites are less than 160 bp apart, the minimum length required for a single nucleosome (Butler, 1984). The sites are not a result of sequence-specific cutting, so cleavage is unlikely to be in the core DNA. (Micrococcal nuclease does cut its preferential sites in ribosomal spacer DNA of D.discoideum when these are within the nucleosome core (Parish et al., submitted), but only after digestion with relatively high enzyme activities). The location of hypersensitive sites was also not consistent with two possible nucleosome phasing patterns. Little cleavage is found between 1300 and 2400 bp upstream, so non-phased nucleosomes could be present in this region. A series of sites with a 200 bp repeat indicate at least partially phased nucleosomes may occur 550 - 1800 bp downstream from the 3' end of the coding region. The results imply that chromatin immediately flanking the coding region is not packed into nucleosomes even when the gene is inactive. In experiments like the one in Fig. 11, we have never measured a ladder longer than 8 (or possibly 9) nucleosomes, although pSC253, a gene

with a longer coding region, gave a ladder of at least 14 nucleosomes.

Repressor proteins bind to the flanking regions of the gene when the DNA replication is occurring and keep these regions in an open configuration. Strictly speaking they are "pre-activation factors", since in their absence the inactive supranucleosomal structure will form. We call them "repressors" because in the model the gene cannot be transcribed even if transcription factors (TFs) are present (see below).

When the cysteine proteinase I gene is activated at about 6 h it is also transcribed. However, the results with 15 h cells show the gene can be in an activated state but not be transcribed. We postulate that the synthesis of activation factors (AFs) is induced around 6 h (possibly by cAMP) and these bind to regions upstream of the gene, displacing the repressor proteins (which may still, however, be associated with the DNA - see below) and thereby changing the pattern of hypersensitive sites dramatically. Before the gene can be transcribed, TFs are required. These recognize the AFs and subsequently RNA polymerase can bind to the complex and transcription commences. At the 6 h stage the requisite TFs are available for the cysteine proteinase I gene. Later in development the concentration of available TFs drops, either because synthesis ceases or other genes are competing more successfully with the cysteine proteinase I gene for them. Nevertheless, the gene remains in the stable, activated state and return to the repressed state may require DNA synthesis (see below). This would explain the observation that when developing D.discoideum cultures are disaggregated the cells are capable of recapitulating the same sequence of previous morphological and biochemical changes in a fraction of the original time (Newell et al., 1971; Soll and Waddell,

1975; Parish and Schmidlin, 1979). The transcription complex of Xenopus 5 S RNA genes has been found remarkably stable in vitro (Brown, 1984; Setzer and Brown, 1985).

The endogenous nuclease could theoretically be a TF since its activity was only detectable when the gene was being transcribed. Cleaving the DNA in the 5' and 3' coding region would change torsional stress and may be important for transcription. However, the endogenous nuclease probably cleaves a number of nucleotides and a single, immediately ligated cut, such as introduced by a topoisomerase, would be preferable. The endogenous nuclease may be recognizing changes in the DNA structure (eg. hairpin-like) which are formed in the protein-free regions of the activated gene when TFs bind to the 5' flanking region and the transcription complex reads into the 3' flanking region. Micrococcal nuclease can cleave the protein-free regions even in the absence of transcription.

When the gene is being transcribed, the transcription complex carries the machinery which switches the nucleosomes of the coding region to the "active", nuclease-sensitive conformation. The nucleosomes rapidly return to the "normal" configuration when the transcription complex has passed, the rate of entry of the complex (density) determining how many "normal" nucleosomes will be associated with the coding region. Thus, nucleosomes reform on the coding region of 15 h cells even though the gene is still in the active state.

The only difference between the activated and transcribed states detected by micrococcal nuclease digestion was the replacement of the 800 and 900 bp upstream hypersensitive sites by a site at 850 bp when the gene was no longer transcribed. In the

downstream region there was one difference when the gene was being transcribed, namely the endogenous nuclease cleaving a site at 250 bp. This site was either not recognized or was rapidly trimmed by micrococcal nuclease. It may always be present but only detectable when the endogenous nuclease is available, or represent a change in DNA secondary structure induced by transcription. It might coincide with a hairpin-like terminator sequence (Birchmeyer et al., 1984).

Recently the genomic clone of the cysteine proteinase I has been isolated (J. Williams personal communication). The flanking sequences can now be sequenced and the nuclease hypersensitive sites precisely characterized.

V. SUMMARY

The cysteine proteinase I gene of Dictyostelium discoideum is a single copy, developmentally regulated gene. We have used micrococcal nuclease digestion of nuclei from various developmental stages to investigate changes in the chromatin structure of the coding region during gene regulation. Following digestion, DNA fragments were separated by gel electrophoresis, transferred to DBM paper and hybridized to a cDNA clone. A typical nucleosome repeat was obtained from nuclei of vegetative cells and early developmental stages where the gene was inactive. When the gene was transcribed, a smear superimposed on the nucleosome ladder was observed. In the late developmental stages when the gene was again inactive, the nucleosomal pattern returned. Probing with cDNA from a gene that is constitutively expressed gave a smear throughout development. Conversely, an inactive gene was always packaged into nucleosomes.

No difference was detected in the accessibility of the cysteine proteinase I gene to restriction endonucleases during development

The 5' and the 3' flanking regions of the gene were cleaved by an endogenous nuclease when the gene was being transcribed. The Indirect end-labelling technique was used to map the cleavage sites. The majority of these sites were not cut when the gene was inactive. The endogenous nuclease may become associated with the gene or recognize changes in the DNA structure occurring during

transcription.

The micrococcal nuclease hypersensitive sites were also mapped by indirect end-labelling. A dramatic change in the pattern of hypersensitive sites occurred in the 5' flanking when transcription commenced at the 6 h stage of development. The major sites corresponded to those cut by the endogenous nuclease. When transcription subsequently ceased the hypersensitive sites did not significantly change, indicating the gene remained in an activated state. Since the coding region was again packaged into nucleosomes at this stage, the transcriptional machinery may transitorily open the nucleosome structure during transcription. The hypersensitive sites in the 3' flanking region did not change significantly during development.

A model is presented relating the changes in chromatin structure to gene activation and transcription.

ZUSAMMENFASSUNG

Das Cystein Proteinase I Gen von Dictyostelium discoideum ist ein entwicklungsreguliertes Einzelkopie-Gen. Die Änderungen der Chromatinstruktur in der kodierenden Region bei der Gen-Aktivierung und -Inaktivierung wurden anhand des Verdauemusters der Micrococcus-Nuklease untersucht. Dazu wurden Zellkerne aus verschiedenen Stadien des Entwicklungszyklus isoliert und mit Micrococcus-Nuclease verdaut. Anschliessend wurden die gereinigten DNA-Fragmente in Agarosegelen aufgetrennt, auf DBM-Papier transferriert und mit einem cDNA-Klon des Gens hybridisiert. In vegetativen Zellen und Frühstadien der Differenzierung, wenn das Gen inaktiv war, konnte das klassische Nukleosomenmuster festgestellt werden. Sobald das Gen jedoch transkribiert wurde, verschwand das normale Nukleosomenmuster, um in den Spätstadien, wenn das Gen wieder abgeschaltet wurde, erneut aufzutreten. Bei der Hybridisierung mit dem cDNA-Klon eines konstitutiv exprimierten Gens, traten während des gesamten Entwicklungszyklus keine distinkten Nukleosomenbanden auf. Im Gegensatz dazu konnte bei einem, bis in die Spätstadien inaktiven, Gen das klassische Nukleosomenmuster beobachtet werden.

In der kodierenden Region des Cystein Proteinase I Gens konnte über den ganzen Entwicklungszyklus keine Änderung der Sensitivität gegenüber Restriktionsenzymen nachgewiesen werden.

Die 5' und 3' flankierenden Regionen des Cystein Proteinase I Gens wurden, sobald die Transkription startete, von einer

endogenen Nuklease an ganz bestimmten Stellen attackiert. Im Gegensatz dazu wurde die Mehrzahl dieser Stellen im inaktiven Gen nicht geschnitten. Diese Schnittstellen wurden mit Hilfe der indirekten Endmarkierungs-Technik lokalisiert. Die endogene Nuklease könnte in irgend einer Form an der Aktivierung des Gens beteiligt sein oder Aenderungen in der DNA Struktur während der Transkription erkennen.

Die Charakterisierung der hypersensitiven Schnittstellen der Micrococcus-Nuklease erfolgte ebenfalls mittels der indirekten Endmarkierungs-Technik. In der 5' flankierenden Region des Gens traten grosse Aenderungen im Muster der hypersensitiven Schnittstellen auf, sobald das Gen eingeschaltet wurde. Die im aktiven Zustand des Gens aufgetretenen Hauptbanden waren mit denjenigen der endogenen Nuklease identisch.

In den Spätstadien, wenn das Gen ausgeschaltet wurde, änderte sich das Muster der hypersensitiven Schnittstellen nicht signifikant, was zur Annahme führt, dass das Gen in einem aktivierten Zustand bleibt. Die Tatsache, dass die kodierende Region des Gens in diesem Entwicklungsstadium wieder in Nukleosomen gepackt war, spricht dafür, dass die Transkriptions-Maschinerie für die Oeffnung der Nukleosomenstruktur während der Transkription verantwortlich ist. In der 3' flankierenden Region des Cystein Proteinase I Gens fanden im Laufe des Differenzierungszyklus keine signifikanten Aenderungen im Muster der hypersensitiven Schnittstellen statt.

Die vorliegenden Daten wurden in einem Modell zusammengefasst, welches die Aenderungen der Chromatinstruktur in Beziehung zur Genaktivierung und Transkription stellen.

VI. REFERENCES

- Alwine, J.C., Kemp, D.J., Parker, B.A., Reiser, J., Renart, J., Stark, G.R. and Wahl, G.M. (1979) *Methods Enzymol.* 68, 220-242.
- Anderson, J.N., Vanderbilt, J.N., Lawson, G.M., Ming-Jer, T. and O'Malley, B.W. (1983) *Biochemistry*, 22, 21-29.
- Banerji, J., Olson, L. and Schaffner, W. (1983) *Cell*, 33, 729-740.
- Barondes, S.H., Cooper, D.N. and Haywood-Reid, P.L. (1983) *J. Cell Biol.* 96, 291-296.
- Birchmeier, C., Schümperli, D., Sconzo, G. and Birnstiel, M.L. (1984) *Proc. Natl. Acad. Sci. USA*, 81, 1057-1061.
- Bogdanovsky-Sequeval, D., Ayala, J., Mathieu, M., Presse, F. and Felsenbok, B. (1984) *Biol. Cell.* 50, 217-222.
- Bogenhagen, D.F., Sakonju, S. and Brown, D.D. (1980) *Cell*, 19, 27-35.
- Bryan, P., Hofstetter, H. and Birnstiel, M.L. (1982) *Cell*, 27, 459-466.
- Brown, D.D. (1984) *Cell*, 37, 359-365.
- Butler, P.J.G. (1983) *CRC Crit. Rev. Biochem.* 15, 57-91.
- Butler, P.J.G. (1984) *EMBO J.* 3, 2595-2604.
- Burch, J.B.E. and Weintraub, H. (1983) *Cell*, 33, 65-76.
- Cartwright, I.L., Abmayr, S.M., Fleischman, G., Lowenhaupt, K., Elgin, S.C.R., Keene, M.A. and Howard, G.C. (1982) *CRC Crit. Rev. Biochem.* 13, 1-86.
- Cereghini, S., Herbomel, P., Jouanneau, J., Saragosti, S., Katinka, M., Bourachot, B., de Crombrughe, B. and Yaniv, M. (1983) *Cold Spring Harbour Symp. Quant. Biol.* 47, 935-944.
- Charlesworth, M.C., and Parish, R.W. (1977) *Eur. J. Biochem.* 75, 241-250.

- Chung, S.Y., Folsom, V. and Wooley, J. (1983) *Proc. Natl. Acad. Sci. USA*, 80, 2427-2431.
- Chung, S., Zuker, C. and Lodish, H.F. (1983) *Nucl. Acids Res.* 11, 4835-4852.
- Chung, S., Landfear, S.M., Blumberg, D.D., Cohen, N.S. and Lodish, H.F. (1981) *Cell*, 24, 785-797.
- Cocucci, S.M. and Sussman, M. (1970) *J. Cell Biol.* 45, 399-407.
- Davis, A.H., Reudelhuber, T.L. and Garrard, W.T. (1983) *J. Mol. Biol.* 166, 361-381.
- DeLotto, R. and Schedl, P. (1984) *J. Mol. Biol.* 179, 607-628.
- Devine, J.M., Tsang, A.S. and Williams J.G. (1982) *Cell*, 28, 793-800.
- Dingwall, C., Lomonosoff, G.P. and Laskey, R.A. (1981) *Nucl. Acids Res.* 9, 2659-2673.
- Fritton, H.P., Sippel, A.E. and Igo-Kemenes, T. (1983) *Nucl. Acids Res.* 11, 3467-3484.
- Fritton, H.P., Igo-Kemenes, T., Nowock, J., Strech-Jurk, U., Theisen, M. and Sippel, A.E. (1984) *Cell*, 311, 163-165.
- Gilles, S.D., Morrisch, S.L., Oi, V.T. and Tonegawa, S. (1983) *Cell*, 33, 717-728.
- Gocke, E., Bonven, B.J. and Westergaard, O. (1983) *Nucl. Acids Res.* 11, 7661-7668.
- Gottesfeld, J.M. and Bloomer, L.S. (1980) *Cell*, 21, 751-760.
- Hofstetter, H., Kressman, A. and Birnstiel, M.L. (1981) *Cell*, 24, 573-585.
- Hörz, W. and Altenburger, W. (1981) *Nucl. Acids Res.* 9, 2643-2658.
- Igo-Kemenes, T., Hörz, W. and Zachau, H.G. (1982) *Annu. Rev. Biochem.* 51, 89-121.
- Jongstra, J., Reudelhuber, T., Oudet, P., Benoist, C., Chae, C.B., Jeltsch, J.-M., Mathis, D. and Chambon, P. (1984) *Nature (London)*, 307, 708-714.
- Kaye, J.S., Bellard, M., Dretzen, G., Bellard, F. and Chambon, P. (1984) *EMBO J.* 3, 1137-1144.

- Keene, M.A. and Elgin, S.C.R. (1980) *Cell*, 27, 57-64.
- Labhart, P., Banz, E., Ness, P.J., Parish, R.W. and Koller, Th. (1984) *Chromosoma*, 89, 111-120.
- Labhart, P., Ness, P.J., Banz, E., Parish, R.W. and Koller Th. (1983) *Cold Spring Harbor Symp. Quant. Biol.* Vol. XLVII, 557-564.
- Levy, A. and Noll, M. (1981) *Nature (London)*, 289, 198-203.
- Lohr, D. (1981) *Biochemistry*, 20, 5966-5972.
- Lohr, D. (1983) *Nucl. Acids Res.* 11, 6755-6773.
- Louis, C., Schedl, P., Samal, B. and Worcel, A. (1980) *Cell*, 22, 387-392.
- Ma, G.C. and Firtel, R.A. (1978) *J. Biol. Chem.* 253, 3924-3932
- Mangiarotti, G., Lefebvre, P. and Lodish, H.F. (1982) *Dev. Biol.* 89, 82-91.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Cold Spring Harbor Laboratory Press NY*.
- Mathis, D., Oudet, P. and Chambon, P. (1980) *Progr. Nucl. Acids. Res. Mol. Biol.* 24, 1-55.
- McGhee, J.D., Wood, W.I., Dolan, M., Engel, J.D. and Felsenfeld, G. (1981) *Cell*, 27, 45-55.
- McKnight, S.L. and Kingsbury, R. (1982) *Science*, 217, 316-325.
- Mills, F.C., Fisher, M.L., Kuroda, R., Ford, A.M. and Gould, H.J. (1983) *Nature*, 306, 809-812
- Nedospasov, S.A. and Georgiev, G.P. (1980) *Biochem. Biophys. Res. Commun.* 92, 532-539.
- Ness, P.J., Banz, E., Parish, R.W. and Koller, Th. (1985) in preparation.
- Ness, P.J., Labhart, P., Banz, E., Koller, Th. and Parish, R.W. (1983) *J. Mol. Biol.* 166, 361-381.
- Newell, P.C., Longlands, M. and Sussman, M. (1971) *J. Mol. Biol.* 58, 541-554.
- Palen, T.E. and Cech, T.R. (1983) *Nucl. Acids Res.* 11, 2077-2091.
- Parish, R.W. and Schmidlin, S. (1985) *Nucl. Acids Res.* 13, 15-29.

- Parish, R.W., Stalder, J. and Schmidlin, S. (1977) FEBS Letters, 84, 63-66.
- Parish, R.W., Banz, E. and Ness, P.J. (1985) submitted.
- Parslow, T.G. and Granner, D.K. (1982) Nature (London), 299, 449-451.
- Picard, D. and Schaffner, W. (1984) Nature (London), 307, 80-82.
- Poole, S.J. and Firtel, R.A. (1984) J. Mol. Biol. 172, 203-220.
- Poole, S., Firtel, R.A., Lamar, E. and Rowekamp, W. (1981) J. Mol. Biol. 153, 273-290.
- Prior, C.P., Cantor, C.R., Johnson, E.M., Littau, V.C. and Allfrey, V.G. (1983) Cell, 34, 1033-1042.
- Queen, C. and Baltimore, D. (1983) Cell, 33, 729-740.
- Rave, N., Crkvenjakov, R. and Boedker, H. (1979) Nucl. Acids Res. 6, 3559-3567.
- Reeves, R. (1984) Biochim. Biophys. Acta, 782, 243-393.
- Rowekamp, W., Poole, S. and Firtel, R.A. (1980) Cell, 20, 495-505.
- Samal, B., Worcel, A., Louis, C. and Schedl, P. (1981) Cell, 23, 401-409.
- Saragosti, S., Moine, G. and Yaniv, M. (1980) Cell, 20, 65-73.
- Saragosti, S., Cereghini, S. and Yaniv, M. (1982) J. Mol. Biol. 160, 133-146.
- Schwinghamer, M.W. and Shepherd, R.J. (1980) Anal. Biochem. 103, 426-434.
- Setzer, D.R. and Brown, D.D. (1985) J. Biol. Chem. 260, 2483-2492.
- Shermoen, A.W. and Beckendorf, S.K. (1982) Cell, 29, 601-607.
- Sogo, J.M., Ness, P.J., Widmer, R.M., Parish, R.W. and Koller, Th. (1984) J. Mol. Biol. 178, 897-928.
- Soll, D.R. and Waddell, D.R. (1975) Dev. Biol. 47, 292-302.
- Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
- Stalder, J., Groudine, M., Dodgeson, J.B., Engel, J.D. and Weintraub, H. (1980) Cell, 19, 973-980.
- Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M. and Weintraub, H. (1980) Cell, 20, 451-460.

- Sweet, R.W., Chao, M.V. and Axel, R. (1982) *Cell*, 31, 347-353.
- Tsang, A.S., Devine, J.M. and Williams J.G. (1981) *Dev. Biol.* 84, 212-217.
- Udvardy, A. and Schedl, P. (1984) *J. Mol. Biol.* 172, 385-403.
- Weintraub, H. (1984) *Cell*, 38, 17-27.
- Weisbrod, S.T. (1982) *Nature (London)*, 297, 289-295.
- Weischet, W.O., Glotov, B.O. Schnell, H. and Zachau, H.G. (1982) *Nucl. Acids Res.* 10, 3627-3644.
- Weischet, W.O., Glotov, B.O. and Zachau, H.G. (1983) *Nucl. Acids Res.* 11, 3593-3612.
- Widmer, R.M., Fuhrer, S. and Parish, R.W. (1979) *FEBS Letters*, 106, 363-369.
- Widmer, R.M., Lucchini, R., Lezzi, M., Meyer, B., Sogo, J.M., Edström, J.-E. and Koller Th. (1984) *EMBO J.* 3, 1635-1641.
- Williams, J.G. and Lloyd, M.M. (1979) *J. Mol. Biol.* 129, 19-35.
- Williams, J.G., Lloyd, M.M. and Devine, J.M. (1979) *Cell*, 17, 903-913.
- Williams, J.G., North, M.J. and Mahbubani, H. (1985) *EMBO J.*, 4, 999-1006.
- Williams, J.G., Tsang, A.S. and Mahbubani, H. (1980) *Proc. Natl. Acad. Sci. USA*, 77, 7171-7175.
- Worcel, A., Gargiulo, G., Jessee, B., Udvardy, A., Louis, C. and Schedl, P. (1984) *J. Mol. Biol.* 11, 421-439.
- Wu, C. (1980) *Nature (London)*, 286, 854-860.
- Zuker, C. and Lodish, H. F. (1981) *Proc. Natl. Acad. Sci. USA*, 78, 5386-5390.

VII. ABBREVIATIONS

AF	Activation factor
bp	base pair
Ci	Curie
cpm	Counts per minute
DBM	Diazobenzylloxymethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
kb	Kilobase
mRNA	Messenger RNA
RNA	Ribonucleic acid
RNase	Ribonuclease
TF	Transcription factor
Tris	Tris(hydroxymethyl)aminomethane
V	Voltage

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Roger W. Parish for his stimulating guidance throughout my work. I am particularly indebted to Elisabeth Banz for her excellent technical assistance. Further I also gratefully acknowledge the generous gift of clones from Dr. H.F. Lodish and Dr. J.G. Williams.

CURRICULUM VITAE

von Jovan Pavlović, geboren am 3. April 1953 in Zürich, Bürger von
Rüschegg Be

- 1960-1966 Primarschule Zürich
- 1966-1969 Sekundarschule Zürich
- 1969-1970 Vorbereitungsklasse für die Mittelschule Institut Juventus
- 1970-1973 Kantonale Oberrealschule Zürich
- 1973-1975 Tagesgymnasium Institut Juventus, kantonale Matura Typus C Zürich
- 1975 Immatrikulation an der Philosophischen Fakultät II der
Universität Zürich. Studium mit Hauptfach allg. Botanik
- 1980 Diplom in allg. Botanik
- 1980-1985 Doktorarbeit unter der Leitung von Prof. R.W. Parish am Institut
für Pflanzenbiologie. Seit 1979 Assistent am gleichen Institut.

Während meines Studiums besuchte ich bei folgenden Dozenten Vorlesungen,
Kurse und Praktika:

Physik: Prof. E. Brun

Physikalische Chemie: Prof. H. Fischer, Prof. H. Labhart, Prof. G. Wagnière,
Dr. W. Heinzelmann

Chemie: Prof. C.H. Eugster, Prof. H.R. Oswald

Mathematik: Prof. E. Batschelet

Biochemie: Prof. P. Christen, Prof. R. Humbel, Prof. J. Kägi
Prof. H.R. Bosshard, PD Dr. A. Jakob, PD Dr. K. Lerch
Dr. P. Sonderegger, Dr. K. Wilson

Molekularbiologie: Prof. M. Birnstiel, Prof. W. Schaffner,
Prof. C. Weissmann, Prof. H. Zuber, Prof. H. Dutler,
Dr. W. Hofstetter, Dr. R. Portmann, PD Dr. H. Weber

Anthropologie: Prof. J. Biegert

Paläontologie: Prof. E. Kuhn-Schnyder, Prof. H. Rieber

Zoologie: Prof. H. Burla, Prof. P.S. Chen, Prof. R. Wehner,
Prof. V. Ziswiler, PD Dr. A. Dübendorfer, Dr. D.C. Turner

Allgemeine und Systematische Botanik: Prof. R. Bachofen, Prof. C.D.K. Cook,
Prof. H. Hohl, Prof. R.W. Parish, Prof. D. Rast,
Prof. H. Wanner, PD Dr. T. Baumann, Dr. G. Dütsch, Dr. W. Egger,
PD Dr. B. Eller, PD Dr. U.P. Roos, PD Dr. H.P. Ruffner,
Dr. K. Schneider

